Christine Helsen
Stefan Prekovic
Bojan D. Petrovic
Thomas Van den Broeck

Editors

PROCEEDINGS

Arandjelovac and Belgrade, Serbia
February 02-08, 2014

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Nanos gigantum humeris insidentes

Preface

Dear colleagues,

The idea of organizing WiBioSE conference came to our minds at the time the UK team was struggling to organize SymBioSE. At first, we thought of organizing SymBioSE instead of the UK team. Luckily team from England managed to organize a smaller event. As lots of people knew about our idea, many were saying that Team Serbia should organize a complementary event. Conversations with my dear friends from Greece (Vasiliki Gerefalaki) and Switzerland (Joëlle Sprunger), as well as some other close people led to the decision that Team Serbia will organize WiBioSE. That is when a new (Winter) Biology Students in Europe (WiBioSE) Conference was born.

Organization of the event was not easy, having in mind the limited time to get everything ready. However, it would not be fair for me to make any comments as I was just someone who started the „landslide", my dear friends and colleagues – Bojan Petrovic, Milica Novakovic, Marina Pekmezovic, Milos Stupar, Jelena Nikolic, Katarina Matijasevic and Katarina Zeljic – were the ones responsible for making everything we had imagined a reality.

The conference will cover numerous topics of biological sciences – from basic biology, ecology, bioarcheology, molecular biology, biochemistry to a more clinically orientated research. The Organizational board has chosen the top specialists from Serbia to give plenary talks and we use this opportunity to thank them, once again, for accepting our offer. Also, the Committee would, specially, like to thank Beatrice Kelemen for coming to Serbia and giving her talk on bioarcheology.

From a scientific standpoint this conference will contribute to the development of young individuals, young scientists, sharing their research and knowledge, experience and opinions. Also, this conference will help us promote science in Serbia (as all of the lectures will be publically accessible) and Serbia as an important player in the scientific arena.

Participants from 14 European countries contributed to our program with their scientific data - in our opinion making WiBioSE Conference a success. With this being said, I hope that WiBioSE will be organized by another country next year and that it will grow and become a big conference.

Enjoy the conference and your stay in Serbia!

President of the Organizing Committee

Stefan Prekovic

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CONFERENCE PROGRAM

	Date	Sunday, 2 February 2014	Monday, 3 February 2014	Tuesday, 4 February 2014	Wednesday, 5 February 2014	Thursday, 6 February 2014	Friday, 7 February 2014	Saturday, 8 February 2014
	Venue	Arandjelovac	Arandjelovac	Arandjelovac	Arandjelovac	Belgrade	Arandjelovac	Arandjelovac
Time	08:00		*Breakfast*	*Breakfast*	*Breakfast*	*Breakfast*	*Breakfast*	*Breakfast*
	09:00		Official opening ceremony (at local "Centar za kulturu") [9:30-10:45]	Session 3	Session 1	Transfer	Session 5	Departure of participants
	10:00							
	11:00		Treasure hunt (cross town)	Excursions (around town) (lunch packet) [10:30-17:00]		Lectures (at University of Belgrade)		
	12:00	Arrival & check in of participants			*Lunch [12:30-13:30]*		Keynote lecture [12:00-12:30]	* Unless specified otherwise, activities are being held at Hotel "Rujna zora", in Arandjelovac
	13:00						*Lunch [12:30-14:30]*	
	14:00	*Buffet lunch* & Registration	*Lunch [14:00-15:30]*		Workshops & Poster session (at local Gymnasium)	Lunch break (lun.pack)		
	15:00		Session 2			Belgrade winter walk (downtown Belgrade)	WiBioSE assembly and Debate: WiBioSE Future	
	16:00	Further arrival/check-in/registration						
	17:00			Session 4			Closing ceremony & Awards (at local "Centar za kulturu") [17:30-18:15]	
	18:00				*Dinner [18:30-19:30]*			
	19:00	*Dinner*	*Dinner [19:15-20:30]*	*Dinner [19:00-20:30]*	WiBioSE games [19:30-21:00]	Conference dinner (Belgrade)	*Dinner [18:45-20:00]*	
	20:00	Get to know each other party					Farewell party	
	21:00		Swimming pool party	Country presentations		Transfer		
	22:00				Night out party			
	23:00							

INVITED SPEAKERS

WHAT WE COULD LEARN FROM THE WONDERS OF CELLULAR WATER – A CAREER PATH IN BIOPHYSICS

Pavle R. Andjus

Center for laser microscopy, Faculty of Biology, University of Belgrade, Serbia

Water is one of the basic and omnipresent molecules in nature. Nevertheless, it is still one of the less understood biological entities with over 60 anomalies. The role of water in biological systems is well known however its structuring in cells is still unknown and its mechanism of transport through cell membranes was discovered only in the 1990's leading to Nobel prize for Peter Agre in 2003 for the discovery of aquaporins or water channels. Today aquaporins are known to be widespread in nature existing in 13 different protein types (AQP 0-10, AgpZ and GlpF). My studies of water started in 1980's when as a young biology graduate I embarked on a project to study mechanisms of water transport through the cell membrane of a Charophyte alga. Working in the Institute of General and Physical Chemistry employing an original NMR technique we discovered an unusual transmebrane water transport temperature dependence with discontinuities at 15 and 30 ^{o}C (Fig. 1). The same critical temperatures were also later confirmed by electrophysiological measurements of the resting membrane potential.

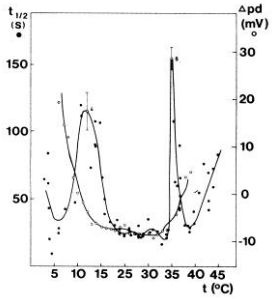

Fig. 1. *Temperature dependence of membrane water transport (full symbols/left ordinate) and membrane potential difference (open symbols/right ordinate) in Chara gymnophylla.*

Differential scanning calorimetry of isolated membranes could reveal a phase transition in these membranes that could explain the low temperature water efflux change. However, the large and sharp transient at 30^{o}C could only be explained by a phase transition in membrane bound water. The latter phenomenon was based on a revolutionary concept of prof. Walter Drost Hansen on the "vicinal water" at interfaces and its thermal transition temperatures conserved in nature [1]. This concept could only be accepted by comparison with analogous studies of water in various biological and non-biological systems. While trying to modify water transport with diverse pharmacological agents we discovered that there was a pathway that had a different pharmacology and temperature dependence than the ion channel pathways that at the time were believed to be also the main passage for transmembrane water. These data

were published in a regional journal [2] and were the basic conclusion of my PhD thesis defended in 1991. In 1993 Peter Agre published his seminal paper naming water channels "aquaporins" [3]. Furthermore, the curiosity of a biologist drove me to the discovery of a massive effect of H_2O substitution with D_2O - the medium in which my colleagues physical chemists performed water transport measurements by NMR. Thus, actually an addition of only one more neutron in the hydrogens of water caused a real excitation of the membrane potential in internodal cells of *Chara* [4]. This further opened a whole series of studies on the importance of the hydrogen bond in diverse aspects of ion channel function [5,6].

After a certain period of career reorientation and a couple of sabbaticals I dwelled in the field of Neuroscience establishing my own lab at the Faculty of Biology University of Belgrade. One of the main topics we studied there (and still do) were the mechanisms of pathophysiology of a devastating motoneuron disease, amyotrophic lateral sclerosis (ALS). Again in collaboration with physical chemists and neurologists employing Magnetic Resonance Imaging on a rat model of ALS, we discovered that these animals had a prominent leakage of the blood-brain barrier (BBB) [7]. Almost at the same time Zlokovic and co-workers published in Nature Neuroscience a study on a mouse model proposing that the BBB leakage is an important early marker in ALS [8]. We have also noticed by MRI edema-like dilations of lateral ventricles in the brain of ALS animals. At that time we have been in touch with the group of Ole P. Ottersen from University of Oslo and were aware of their studies of AQP4 overexpression in brain edema. I thus suspected that a similar role could be ascribed to AQP4 in ALS. Moreover, from previous studies of the Ottersen group as well as from others we expected also a change in the expression of a transporting protein closely expressed with AQP4 – potassium inward rectifier channel 4.1 (Kir4.1). Indeed, we confirmed by immunohistochemistry, Western blot as well as by electrophysiology on astrocytes (forming cellular "end feet" at the BBB) that AQP4 was overxpressed while Kir4.1 was down regulated in ALS [9]. Thus, turning back to the same molecule - aquaporin but in a completely different milieu lead to a molecular mechanism for the pathophysilogy (BBB leakage) of a devastating disease and to possible therapeutic targets.

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THE ROLE OF VASOPRESSIN IN THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS ACTIVATION DURING THERMAL STRESS

Nebojsa Jasnic, Predrag Vujovic, Iva Lakic, Sinisa Djurasevic, Tanja Jevdjovic, Tamara Dakic, Jelena Djordjevic

Institute of Physiology and Biochemistry, Faculty of Biology, University of Belgrade, Studentski trg 3, 11000 Belgrade, Serbia

INTRODUCTION

Changes in environmental temperature are very common threats, which have to be exceeded in the process of successful acclimatization of every endothermic organism. Previous studies have shown heat to be one of the most intense stressors when compared to cold, immobilization, crowding and fasting, as indicated by the highest rise of plasma adrenocorticotropic hormone (ACTH) and corticosterone (CORT) concentrations following the exposure of animals to high ambient temperatures [1,2]. Additionally, exposure to heat, unlike cold, represents not only an unpleasant experience, but a threat to the osmotic homeostasis as well. Rats, which are known as one of the most successful mammals in coping with extreme environmental conditions [3], are mostly used experimental model for studying these coping strategies. Their first reaction to the thermal stressors is primarily linked with sympatho-adrenomedullary system. However, when aversive stimuli cannot be controlled by this reaction, animals respond by the activation of the hypothalamic-pituitary-adrenal (HPA) axis. In spite of a great deal of information regarding the role of HPA axis in stress response, to date a question remains how the activity of the HPA axis can be specifically regulated. According to some authors [4,5], ACTH response to stress is dependent on the interaction of both, corticotropin releasing hormone (CRH) and vasopressin (VP) secretion. VP may act as an important modulator of ACTH response to stress by potentiating the stimulatory effect of its major regulator, CRH [5-7]. It is widely accepted that parvocellular VP expression and secretion may be independent on the osmotic status, being increased during stress in order to potentiate ACTH release. On the other hand, VP of magnocellular origin is responsible for water conservation and regulation of its secretion depends on osmotic status [8]. However, the previous studies questioned this theory proposing that magnocellular VP could act as secretagogues for ACTH as well, reaching the anterior pituitary via several vascular pathways [9]. It was shown that VP binds to three G protein-coupled receptors, among which V1b receptors are primarily located at the corticotroph surface regulating ACTH secretion [10,11].

Having said all that and owing to the lack of data regarding the control of the HPA axis activation under the heat and cold exposure we designed a series of experiments in order to confirm that mentioned stressors caused an alterations/elevations in concentration of hormones that represent stress markers (ACTH, CORT), as well as in VP levels. After that was confirmed, the next step was to prove that VP was present at the corticotroph membrane during stress exposure. Based on the data obtained from these experiments, we further questioned if VP directly influenced the ACTH secretion.

To answer this question, we employed a selective, non-peptide and orally applicable V1b receptor antagonist, Nelivaptan. Finally, having in mind that paraventricular nucleus (PVN) and supraoptic nucleus (SON) of hypothalamus are the main sources of VP and that their neurons are specifically activated by various stressors, we were wandering if there was a difference in VP amount between these two nuclei.
To achieve these goals, we used male rats of Wistar strain (*Rattus norvegicus*), divided into appropriate experimental groups (as will be shown in the Results section) and various experimental methods including ELISA, Western blott analyses and immunohystochemistry.

RESULTS AND DISCUSSION

The blood ACTH and VP levels expectedly increased during stress exposure, showing time dependency. This was in agreement with morphological and stereological changes we obtained, suggesting that pre-synthesized hormone was secreted at the beginning of exposure, while *de novo* synthesis of ACTH was needed for persistent elevation of circulating hormone.

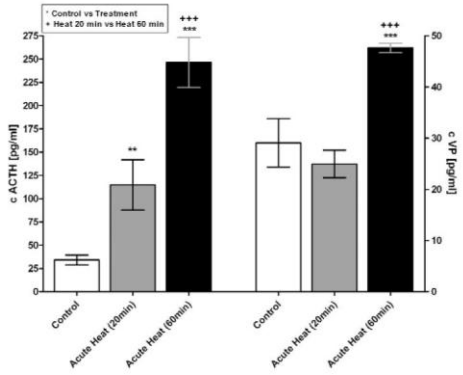

Fig. 1. *The effect of acute exposure of rats to heat (38°C) on ACTH and VP concentration in the blood; **p<0.01, ***p<0.001, +++p<0.001.*
From: *Jasnic at al. Folia Histochemica et Cytobiologica, 2010, 48: 507-12.*

Observed time-dependency regarding circulating VP levels is confirmed by immunohistochemistry, where the VP containing varicose fibers were more numerous in the control and in the 60 min heat exposed animals than in the 20 min treated group. Besides, in the 60 min heat exposed animals the varicose fibers were much thicker, indicating their increased secretory activity.

Further, we observed the strong immunopositivity in plasma membrane of anterior pituitary cells, as well as in blood vessel cells (Fig. 2D). This result supports the role of blood vessels in conducting of VP to the anterior pituitary, where it acts as ACTH secretagogues. Data like this haven't been previously shown by routine immunohistochemistry.

Concluding this part of data presented, it can be said that acute exposure to high ambient temperature induced significant morphological changes in both, anterior and posterior pituitary, accompanied by the elevation of circulating ACTH and VP. These changes were dependent on the duration of heat exposure. Moreover, the VP presence in anterior pituitary strongly suggests its role in potentiating of ACTH secretion.

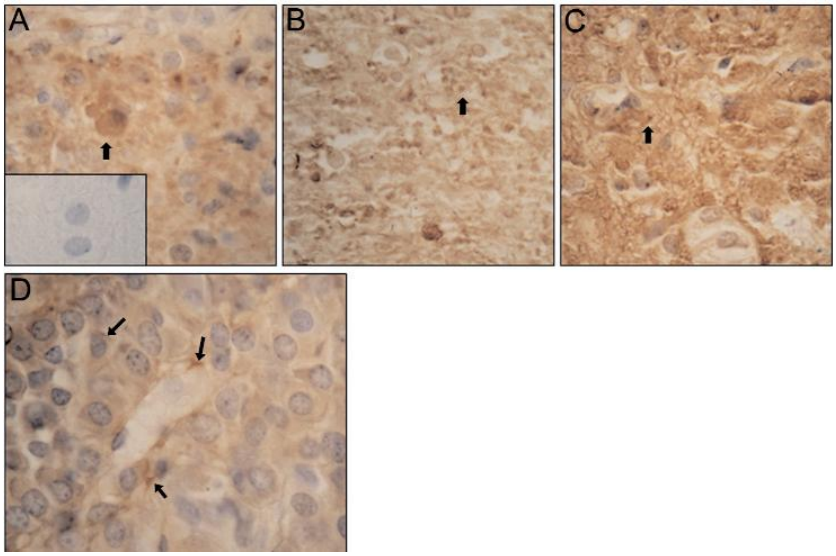

Fig. 2. *VP containing varicose fibres in the posterior pituitary glands of the control (**A**), 20 min (**B**) and 60 min heat exposed rats (**C**) and in the anterior pituitary gland (**D**). Arrows indicate VP immunopositivity of pituitary plasma membrane, as well as blood vessel cells (**D**). Original magnification (A-D) – ×100, inset – nonimmune control.*
From: *Jasnic at al. Folia Histochemica et Cytobiologica, 2010, 48: 507-12.*

Further, we were wondering if VP directly influenced the ACTH secretion, given that it was present in the anterior pituitary. To answer this question, we used a V1b receptor antagonist, Nelivaptan, in combination with exposure to high ambient temperature, which elicited the highest increment of ACTH. As Fig. 3 depicts, the circulating ACTH levels were elevated in animals exposed to heat stress, regardless of the presence of Nelivaptan, while vehicle or Nelivaptan administration did not significantly influence the plasma concentration of this hormone. It is very important to emphasize that the increase in circulating ACTH concentration was significantly attenuated in animals that were pre-treated with Nelivaptan before heat exposure compared with those exposed to heat only. This represents a direct proof of the influence of VP on HPA activity in animals exposed to high ambient temperature.

As mentioned earlier, it is widely accepted that parvocellular VP expression and secretion may be independent on the osmotic status and increased during stress in order to potentiate ACTH release. On the other hand, VP of magnocellular origin is responsible for water conservation and regulation of its secretion depends on osmotic status. Since PVN is consisted of magnocellular and parvocellular neurons, while SON contains exclusively the magnocellular neurons, we investigated potential differences in VP amount in these two hypothalamic nuclei during exposure of animals to low or high ambient temperature.

The obtained results (Fig. 4 and 5) showed that both exposure to high and low ambient temperature increased hypothalamic VP level, although with different intensity since the level was higher under heat conditions. On the other hand, pattern of VP level changes in PVN and SON was stressor-specific, given that cold exposure mainly increased the SON VP level, while heat exposure mostly affected the PVN VP content.

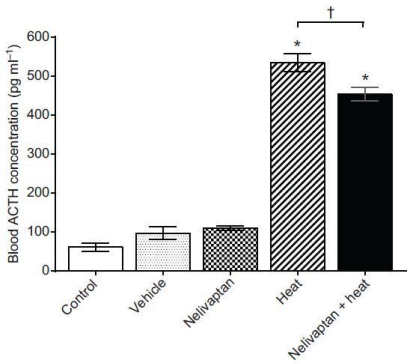

Fig. 3. *Blood ACTH concentration in control animals, and animals treated with vehicle or Nelivaptan, exposed to heat or treated with Nelivaptan prior to heat exposure. An asterisk indicates a significant difference between the control and treated group of animals, while a dagger indicates a significant difference between treated groups. Values are means ± s.e.m. of six animals.*
From: *Jasnic et al. The Journal of Experimental Biology 2013, 216: 2302-7.*

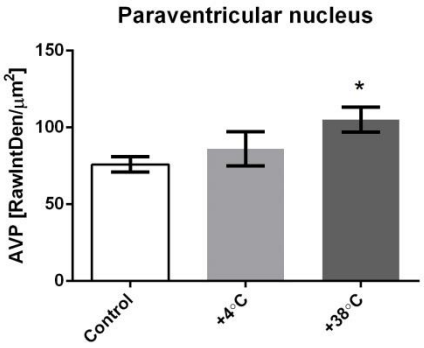

Fig. 4. *VP amount in paraventricular nucleus of control rats as well as those exposed to low and high ambient temperature. Values are means ± s.e.m. of four animals. An asterisk represents the difference between control and treated group of animals.*

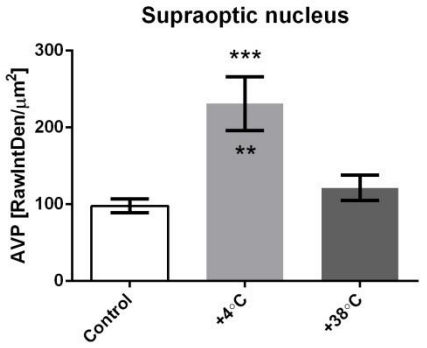

Fig. 5. *VP amount in supraoptic nucleus of control rats as well as those exposed to low and high ambient temperature. Values are means ± s.e.m. of four animals. An asterisk above the bar represents the difference between control and treated group of animals, while asterisk inside the bar represents the difference between two treated groups of animals.*

Taking all described experiments together, the overall conclusion is that cold and heat, as temperature stressors elicit stressor-specific changes in the HPA axis activity and VP secretion. Further, VP reaches the pituitary corticotrophes potentiating ACTH release acting on V1b receptors. However, question still remains how exactly VP reaches anterior pituitary; through the long and short portal vessels, directly through tissue or

through general circulation. Finally, although our results directly confirmed the positive correlation between temperature stressors and changes in hypothalamic VP content, it is still unknown whether this VP originates from parvocellular or magnocellular neurons, which are differently activated upon exposure to various kinds of stressors.

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ABIOTIC STRESS IN BRYOPHYTES AND MECHANISMS OF DEFENSE

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Bryophytes (mosses, liverworts and hornworts) are the non-vascular early evolved land plants spread worldwide from the tropical rain forests to regions with harsh environmental conditions such as desserts or Antarctic. The mechanisms plants use to adapt to abiotic stress have been widely studied in a number of plants. As sessile organisms, mosses are to an extremely high degree affected by changes in the environmental conditions. To counteract the damaging effects of stress situations plants have developed specific molecular and biochemical mechanisms [1-4].

Most bryophytes are desiccation-tolerant organisms, and can survive in the air-dried state for long or short periods even at relative water contents below 10%. Although reactive oxygen species (ROS) are produced during normal metabolism, especially in chloroplasts and mitochondria, desiccation greatly enhances their production. Oxidative stress in various crop plants has been reported in response to salinity, drought, high temperature and pollutants. These toxic ROS cause damage to various cell structures.

Removal of ROS produced during desiccation by antioxidants is also important. Antioxidants include molecules such as the tri-peptide glutathione, ascorbic acid or tocopherols and the enzymes. Plants protect cell and sub cellular systems from the cytotoxic effects of the ROS with antioxidant enzymes superoxide dismutase, glutathione reductase, monodehydroascorbate reductase, catalase.

The ABA-mediated reactions of higher plants are much better understood than those of lower plants, the reactions of higher plants include stomata [5,6]. The knowledge of ABA functions in bryophytes has been limited in comparison of its function in vascular plants [7]. However, ABA is widely known to be one of tracheophyte growth regulator, also known as an universal stress hormone, with a stress dependent biosynthesis, transport to target cells and an action that enables the plant to cope better with the stress situations. In mosses, ABA plays general role in growth inhibition, sex determination and desiccation stress tolerance.

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STEM CELLS AND MODERN MEDICINE

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In the last 30 years knowledge of hematopoietic, mesenchymal, embryonic and induced pluripotency stem cells (iPSC) has evolved rapidly; their potency and differentiation pathways, immunomodulatory properties and paracrine interactions with specific cell types in damaged tissues and promising results in some clinical applications have made these cells an attractive option for the treatment of certain diseases [1]. The growing optimism regarding stem cell research is based on the promising results obtained in *in vitro* and *in vivo* systems. Making use of this range of systems and approaches, recent advances have allowed progress to be made in understanding several key issues that are common to natural regenerative events. These issues include: the determination of regenerative capacity; the importance of stem cells, dedifferentiation and transdifferentiation; how regenerative signals are initiated and targeted; and the mechanisms that control regenerative proliferation and patterning of both exogenous and endogenous stem cell sources [2]. Combined with other technologies previously used to study different model diseases, iPSC modelling has the promise to influence modern medicine on several fronts: early diagnosis, drug development and effective treatment [3]. The rapid translational research with different stem cell sources necessitated standardization of methodology and terminology and greater focus on other aspects such as bio-safety and cellular production, especially for clinical use of stem cells [4]. In addition, many questions regarding the application of stem cells remain unanswered, particularly tumorigenicity, immune rejection and danger of gene manipulation.

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VITAMIN D ANTICANCER PROPERTIES - HYPE OR HOPE

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Forty-three years have passed since the American president Richard Nixon officially announced the "war on cancer". In the previous period great efforts have been made in elucidating the molecular biology of the cancer cell. However, cancer still presents the leading cause of death in the developed world according to the World Health Organization data. Despite significant improvement in diagnosis and treatment, there is a need of discovering and identifying novel prognostic and predictive biomarkers.

Vitamin D deficiency is registered in the majority of human populations worldwide [1]. Previous epidemiological studies reported that insufficient vitamin D concentration is associated with the risk of development of various cancer types and poor patients' prognosis [1,2]. Apart from the well-known function in calcium and phosphorus metabolism and role in bone mineralization, numerous *in vitro* and *in vivo* studies demonstrated anticancer effects of vitamin D [1,2]. Vitamin D is involved in a wide variety of biological processes, including regulation of cell proliferation, differentiation and apoptosis [1-4]. Namely, vitamin D anticancerogen functions are reflected in cell cycle arrest, induction of cell differentiation, apoptosis induction, inhibition of malignant cell invasion and antiangiogenic effect [1-4]. Revealing the potential use of vitamin D supplementation in cancer treatment gave huge hope for patients suffering from different cancers. The question is whether the use of vitamin D in cancer treatment is reasonable hope or it is just hype? In order to find the answer to the previous question it is important to understand molecular level of vitamin D functioning as well as role of genetic and epigenetic changes in vitamin D related genes in cancer.

Vitamin D exerts its functions by binding to the vitamin D receptor (VDR), which is coded by the VDR gene [5,6]. The key enzymes involved in vitamin D metabolism belong to the cytochrome P450 protein family: 1α-hydroxylase, involved in anabolism (coded by CYP27B1 gene) and 24-hydroxylase, involved in the catabolism (coded by the CYP24A1 gene) [5,6]. A great number of single nucleotide polymorphisms (SNPs) are identified in the promoter region of VDR, CYP27B1 and CYP24A1 genes, as well as various exons and introns [6]. Genetic variants in genes related to vitamin D are associated with cancer risk and patients' survival in a number of different tumor types, such as breast, prostate, oral and colorectal cancer [1-6]. In addition, recent studies provide evidence that epigenetic changes could also affect the expression of VDR and vitamin D related genes during carcinogenesis [5]. Epigenetic modifications are mitotically heritable changes in gene expression which are not coded in the DNA sequence. The main mechanisms of epigenetic regulation in mammals are DNA methylation, histone modifications and RNA silencing. These changes have recently been linked to genes related to vitamin D metabolism [5]. Although some of these issues remain unrevealed,

elucidating the mechanisms of genetic and epigenetic changes in vitamin D related genes may serve as a tool to predict an individuals' susceptibility to cancer, or provide dietary recommendations of vitamin D against cancer.

The present plenary lecture gives a comprehensive overview of the vitamin D anticancer properties, as well as possibility and challenges of potential using of vitamin D and/or vitamin D synthetic analogs for preventive and therapy purposes.

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BIOLOGICAL CONTROL OF PLANT PATHOGENS BY THE STRAINS OF *BACILLUS* SP.

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SUMMARY

Bacteria from genus *Bacillus* are microorganisms that inhabit many different ecological niches. They produce a number of secondary metabolites, including nonribosomally synthesized peptides and lipopeptides, polyketide compounds and bacteriocins. Lipopeptides from *Bacillus* have very complex mechanisms of biosynthesis and in general have a broad spectrum of antagonistic activity against plant pathogenic bacteria, fungi and viruses. Most important molecules from this group, surfactins, iturins and fengycins affect the target cells on membrane level, acting as biosurfactants or by formation of ion-conducting pores. *Bacillus* strains exhibit their biocontrol capacity predominantly through antibiosis as well as inducing systemic resistance in plants and competing for ecological niches with plant pathogens. These properties qualify strains of *Bacillus* as powerful tools in biological control of plant pathogens. Our recent study indicated frequent presence of biosynthetic operons for synthesis of nonribosomal lipopeptides in natural isolates of *Bacillus* with many strains having more than one of them. Two analyzed strains showed ability to inhibit the growth of several fungal and bacterial plant pathogens, thus proving to be good candidates for biocontrol agents.

INTRODUCTION

In last decade, the interest for biological control of plant pathogens has increased, particularly because of the necessity to introduce environmentally acceptable alternatives to the extensive use of chemical pesticides [1]. Besides that, the strategies for combating crop diseases in many cases have to include alternatives to chemical pesticides, since the conventional approach in pest control cannot give satisfactory results. The application of beneficial microorganisms as biocontrol agents is therefore considered as one of the most promising methods for more efficient and safe crop protection. Among microorganisms used for that purpose, bacteria from genera *Bacillus, Pseudomonas* and *Agrobacterium* play the most important role [2]. Compared to the others, bacteria from genus *Bacillus* have numerous advantages. Beside the few exceptions, most of the *Bacillus* species are considered as safe microorganisms, and they have ability to synthesize an incredibly wide array of secondary metabolites with strong antifungal and antibacterial activity. In general, members of *Bacillus* sp. inhabit the soil, but they can also exist as epiphytes and endophytes in the plant rhizosphere and spermosphere. In addition, they grow very fast in different media and form heat-resistant spores, which make them the best candidates for biocontrol applications.

Diversity and distribution of bacteria from genus Bacillus

Bacteria from genus *Bacillus* are aerobic, Gram-positive, endospore-forming, catalase positive microorganisms, represented by a large number of species and wide

distribution in different ecological niches. In the latest edition of Bergey's Manual of Systematic Bacteriology [3] 142 species were included in genus *Bacillus*. Bacilli are present in an extremely large palette of environments, and they can be found in soil, sea or sweet water, on the surface of plants, in foods and in gastrointestinal tract of mammals and other animals. Most species of the genus *Bacillus* have little or no pathogenic potential and are rarely associated with disease in humans and other animals; an exception is *Bacillus anthracis*, the agent of anthrax; several other species may cause food poisoning and opportunistic infections, and *Bacillus thuringiensis* are pathogenic to invertebrates, feature used in biocontrol [3]. Revealed diversity within Bacilli indicates potential for certain genotypes/phenotypes related to biological control, also. Knowledge of the diversity within a group of strains that share a common biocontrol trait may provide a new approach to identify biocontrol strains that are superior with respect to ecological competence and ability to suppress specific plant diseases.

Antimicrobial substances in Bacillus species

Among other characteristics of *Bacillus* species, one of their most important features, particularly from biotechnological point of view, is their ability to produce wide array of antimicrobial substances. *B. subtilis* has an average of 4-5% of its genome devoted to antibiotic synthesis and has the potential to produce more than two dozen structurally diverse antimicrobial compounds [4]. In strain FZB42, which is proposed as a paradigm for plant-associated *B. amyloliquefaciens* as well as in other isolates, an even larger part of the genome (~8%) is seemingly involved in antibiotic synthesis. These compounds can have very diverse chemical structure, and include antimicrobial peptides and lipopeptides, as well as bacteriocins.

Nonribosomal peptides and lipopeptides are heterogeneous group of compounds, consisting of amino acids, hydroxyl or amino fatty acids, very often modified by methylation, acylation or glycosilation. These peptides are synthesized by nonribosomal peptide synthetases (NRPSs), large enzyme complexes with modular structure, with each module being in charge for the incorporation of particular amino acid [5]. The initial reaction of the biosynthesis is the transfer of phosphopantetheinyl group to the PCP (peptidyl carrier protein), catalyzed by the specific phosphopantetheinyl transferase. The modules are consisting of domains, starting with A domain, which catalyzes activation of amino acid by adenilation. Activated amino acid is then transferred to the 4'-phosphopantetheine group of PCP, forming a thioester bond. C or condensation domain catalyzes the formation of peptide bond between amino acids during the synthesis of the peptide. The modules also may contain the E module, which is in charge for epimerization of amino acids, since nonribosomal peptides contain D and L stereoisomers of amino acids.

Mechanisms of action of lipopeptides are mostly based on their amphiphilic nature and their capacity to interact with the cell membrane. The surfactin family consists of very powerful biosurfactants that readily associate and tightly anchor into lipid layers and can thus interfere with biological membrane integrity in dose-dependent manner causing in high concentration irreversible pore formation and complete disruption and solubilisation of the lipid bilayer [1]. The mechanism of interaction of surfactin with biological membranes has been the object of numerous studies. The initial step of surfactin action on the cell membrane is based on hydrophobic interactions with the hydrocarbon chains of membrane lipids, destabilizing the structure of the membrane and its thickness. The first interaction with the membrane is then followed by the conformational changes of the cyclic peptide part, further facilitating the integration into the cell membrane. According to this model, the membrane is destabilized by the

integration of surfactin dimers into the bilayer [6]. Biological activity of the iturins is different to surfactin. Their cytotoxicity relies on their membrane permeabilization properties through osmotic perturbation e.g. formation of ion-conducting pores, not membrane disruption or solubilisation [7]. Fengycins also readily interact with lipid bilayer and to some extant retain the potential to alter cell membrane structure and permeability.

RESULTS

Screening of 203 *Bacillus* sp. natural isolates for antimicrobial activity against phytopathogenic bacteria showed that 127 tested strains inhibit at least one sensitive strain, which illustrates their potential for use as biocontrol agents. Among them, 104 isolates showed significant antagonism against *Xanthomonas oryzae* pv *oryzae*, and only one of these (VPS50.2) synthesizes bacteriocin. An additional screening tested whether 51 isolates contained genes involved in biosynthesis of lipopeptides of the iturin and surfactin classes. Results showed that 33 isolates harboured the operon for iturin biosynthesis, since six of them carry the *sfp* gene, responsible for the biosynthesis of surfactin. Lipopeptide purification from the supernatant of isolate SS12.9 (identified as *B. subtilis* or *B. amyloliquefaciens*) was performed using ethyl acetate extraction, ultrafiltration and reverse phase HPLC. Mass spectrometry analysis confirmed that isolate SS12.9 produces substance from iturin class with potential for biocontrol of *X. oryzae* pv *oryzae*. [8].

In our further work, A collection of 205 natural isolates of *Bacillus* was tested for presence of genes for biosynthesis of antimicrobial lipopeptides iturin, surfactin, fengycin and bacillomycin D. For detection of iturin producers, in PCR screening we used forward ITUP1-F and reverse ITUP2-R primers, which are able to detect a 2-kb region that includes the intergenic sequence between *ituA* and *ituB* genes. A 675-bp fragment from the gene *sfp* from *B. subtilis* encoding 4'-phosphopantetheinyl transferase, involved in the biosynthesis of surfactin was targeted for amplification by using primers P17 and P18. Other two pairs of primers were BACC1F and BACC1R for bacillomycin D and FEND1F and FEND1R for potential fengycin producers, respectively. The results of the screening showed that majority of tested strains have more than one biosynthetic operon, since 81% possess the genes for bacillomycin D production, 54% for surfactin, 38% for iturin and 25% for fengycin production [9].

Analysis of two strains of *Bacillus* sp., SS-12.6 and SS-13.1, that showed very strong antibacterial and antifungal activity against phytopathogens. The PCR analysis showed that both strains have the genes for biosynthesis of iturin, bacillomycin and surfactin. Kinetics of production of antimicrobial substances in these strains showed that synthesis started at the beginning of exponential phase of growth. Maximum of activity was slowly reached at the beginning of stationary growth phase and was maintained until the end of observed period. Ethyl acetate extracts of cell-free supernatants of both strains were particularly active against several postharvest fungal pathogens, *in vitro* and *in vivo*, in the experiment with apple fruits. Mass spectrometry analysis of ethyl acetate extract of the supernatant of strain SS-12.6 confirmed the presence of antimicrobial lipopeptide surfactin [10].

In our studies of bactreiocins, the strain *Bacillus licheniformis* VPS50.2 was identified as bacteriocin producer, which synthesized the molecule with antimicrobial spectrum that included a significant number of bacteria, such as *Listeria monocytogenes*, meticillin-resistant *Staphylococcus aureus* and β-hemolytic streptococci. Kinetics of bacteriocin synthesis showed that the production started at the transition from exponentially to stationary phase (after 14 h of growth), reached maximum activity at the late stationary phase (after 36 h of incubation) and remained at same level until the end of period of observation. The start of bacteriocin production coincides with the beginning of sporulation. The bacteriocin activity was insensitive to lysozyme and proteinase K, showed partial sensitivity to trypsin and was completely sensitive towards pronase E. Bacteriocin was heat stable after incubation at 100°C for 30 min and retained 70% activity after being autoclaved at 121°C for 15 min. The bacteriocin was stable in wide pH range (pH 2–12) and after the storage for six months at 4°C. The bacteriocin was purified by ammonium sulfate precipitation, chloroform extraction and ultrafiltration. MALDI TOF TOF mass spectrometry of purified sample detected the protein with molecular mass of 3253.209 Da. N-terminal sequencing of purified protein recognized first 15 amino acids with the sequence W E E Y N I I X Q L G N K G Q. BLAST similarity search did not found any match with proteins described so far from NCBI database. Considering these findings, we named the newly characterized bacteriocin as licheniocin 50.2 [11].

CONCLUSIONS AND PERSPECTIVES

Understanding and characterizing the bioactive compounds and optimizing their production in fermentation can increase the efficacy and consistency of a biopesticide in a way that is more comparable to that of synthetic pesticides, but their use will probably raise some questions around residues on food and potential of resistance development. Major regulatory agencies already require toxicology tests on microbes and their bioactive compounds, especially if the latter are included in the end product. The risk of resistance development can be considered as low if the mode of action is based on a combination of several bioactive compounds and sometimes also the living microorganisms, along with their physiological interaction with the target pest. Microorganism biodegradability alleviates possible environmental and non-target concerns, but also results in a lack of persistent activity. Such persistence has been demonstrated with some specifically selected microbial antagonists of fungal diseases applied to foliage. Ecological studies have now demonstrated that microbial biocontrol agents can be rhizosphere or phyllosphere competent, and therefore future advances in biopesticide delivery could be achieved through formulations that improve microbe establishment in these zones. Microbial seed treatments have been used for disease control and for nematode control and are also under consideration for insect control. Coating seeds with biopesticides is an inexpensive option that allows targeted delivery and potentially enhances rhizosphere colonization, but this delivery option requires improved efficacy of coating materials and technology. Having that in mind, the genetic and biochemical characterization of plant-associated *Bacillus* strains is of utmost importance for the development of green pesticides and identification of new biocontrol and plant growth promoting strains.

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INVASIVE MUCORMYCOSIS IN PATIENTS WITH NEOPLASM IN SERBIA – REPORT OF TWO CASES

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INTRODUCTION

Invasive mucormycosis (IMM) is the third most frequent fungal infection in patients with malignancies often resulting in fatal outcome [1].

CASE REPORT 1

A 52-year-old male was diagnosed with acute myeloblastic leukemia. Following febrile neutropenia chest computerized tomography (CT) showed soft-tissue consolidation change. Bronchoscopy and histology indicated aspergillosis. Galactomannan (GM) test showed low positivity and voriconazole was included. After two months, the patient developed a fever and the chest multislice CT showed soft-tissue mass. Voriconazole was reintroduced and bronchoscopy was repeated but new histological and mycological examination confirmed pulmonary IMM, fungus *Rhizopus oryzae*. Amphotericin B (AmB) was started and the complete remission was verified (Fig. 1).

CASE REPORT 2

A 53-year-old male was diagnosed with neoplasm Sqamocellulare oesophagi. Following the induction chemotherapy he developed febrile neutropenia. Patient was admitted to the clinic with hemorrhagic-necrotic lesions of the zygomatic, orbital and frontal regions of the face and with lung infiltrates (CT). Facial necrotic changes were spearheaded expansively and vital parameters were worsened. Mycological examination indicated IMM. AmB therapy was included resulting in favorable outcome (Fig. 2).

CONCLUSION

As a rare and emerging disease IMM is not well understood by the medical community. An improvement of education about prevention, timely diagnosis and proper treatment is necessary.

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Fig. 1. *Clinical finding of patient with IMM after 1st, 7th and 14th day after starting with Amphotericin B therapy.*

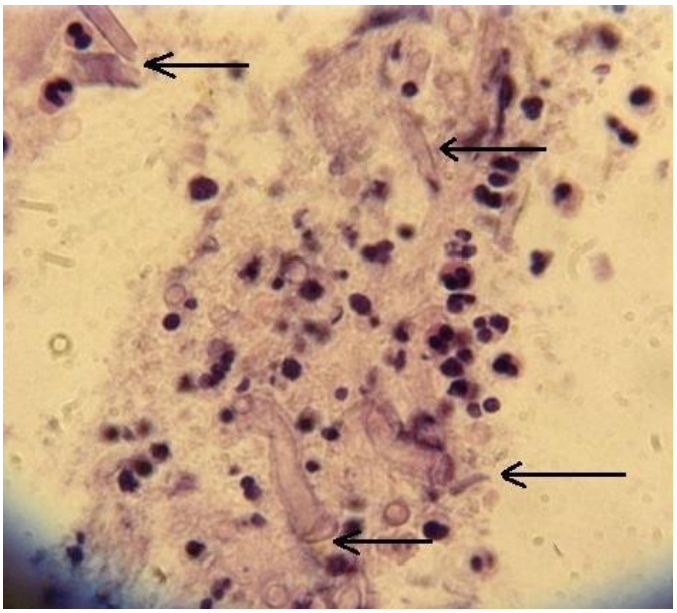

Fig. 2. *Cytology findings of pulmonary tissue: wide nonseptic hyphae, suggesting filamentous fungi of the order Mucorales (Hematoxilin & Eosin staining x 400) (marked with arrows).*

POTENTIAL ROLE OF THE IMUNNE SYSTEM IN THE GENESISS OF GBM INITIATING AND/OR STEM CELLS

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Despite decades of clinicians and scientists efforts brain tumour: glioblastoma multiforme (GBM) remains one of the most lethal malignomas characterized by rapid growth, widespread invasiveness, and intense resistance to all forms of therapy. Current therapy for GBM is almost same as 30 years ago, based on unspecific cytoreductive regimen: surgery, radiation and chemotherapy, while the median survival for GBM patients remains 14.6 months, in Serbia even less. Evidence that GBMs contain tumorigenic stem-like cells gave new hope that more effective therapy approach will result from targeting of this cell subpopulation. Few recent studies suggest that all gliomas including GBM may have origin and is highly dependent on relatively small subpopulation of cells which posses crucial stemness-related characteristics; including self-renewal ability, ability for multipotent differentiation and unfortunately, the ability to resist current forms of therapy. Many authors published papers where they show that this GBM "stem cells" share many morphological and functional neural stem cell-like properties. Furthermore, mouse models have demonstrated that neural stem or neural/glial progenitor cells can give rise to tumors that recapitulate the histopathological hallmarks of human gliomas. Surprisingly, Walton et al. have reported that intermediate progenitors derived from neural stem cells during normal differentiation exibit multiple characteristics of tumor cells such as aneuploidy, loss of growth-contact inhibition, disruptions in cell cycle control and aberrant proteins expression [1]. On the other hand, it was shown that certain subtypes of aggressive gliomas resemble key stages in neurogenesis and implicates signaling pathways that play critical role in the forebrain neurogenesis.

For more than century, titanic travails have been put in order to elucidate the cause of this most common and deadly brain tumor. Some of the accused were variety of viruses, chemicals, different doses of irradiation; however, no strong evidence to date supported any of these. With development of molecular sciences, genetics proved multiple and almost necessary mutations that were governed by certain subtypes of GBM, but reason for this phenomena to arise have not been elucidated yet.

Having all this in mind, it may be useful to take a closer look in the neural stem niche (NSC) and inside explore for some of the reasons for initiation of brain tumors. In the post-mitotic human CNS NSC niche resides in the hypocampal dentate gyrus and subventricular zone (SVZ). Endothelial cells, blood vessels and the basal lamina are all likely key components of the niche as well as in other organs. A specialized basal lamina extends from perivascular cells and contacts all cell types. SVZ "astrocytes" in this region are actually NSC which generate migrating neuroblasts destined for the olfactory bulb via a rapidly dividing transit-amplifying cell. Multi-ciliated ependymal cells line the walls of the lateral ventricle. Chains of neuroblasts travel through tunnels formed by processes of SVZ astrocytes and microglia. Transit-amplifying cells are also found in

small clusters adjacent to the chains. Signals released from axons and microglial cells in tunnel regulate proliferation and survival or apoptosis in this region.

Considering that microglia are adapted to the requirements of their local micro-environment, a unique microglial phenotype is likely associated with the adult neurogenic niche. Furthermore, it must be always considered that any role of microglia in the self-renewing capacity of the CNS, and in the level of proliferation and the differentiation of NSC is to a large extent context dependent, where impact of both local and systemic factors (physiological, or pathological) may play important part of it. Various lines of evidence support the idea that even in health, microglia residing in the adult neurogenic niches bear a special phenotype. It has been shown that microglia associated with the SVZ exist at a higher basal level of activation, based on the expression levels of CD45, CD11b, and IB4. In addition it was shown that acutely activated "activated" SVZ microglia had reductive properties neuronal cell differentiation and increase cell death or astrocyte differentiation through the expression of IL-1α, IL-1β, inducible nitric oxide synthase, IL-6, and TNF-α, while chronically activated microglia became permissive for neurogenesis due to the up-regulation of IL-10 and prostaglandin E2. If there is state severe or moderate chronic inflammation and/or inadequate immune response (nevertheless local or systemic because above mentioned cytokines are small enough to easily overcome blood-brain barrier) the turbulences in NSC niche might be vast, tolerating more or less differentiated cells in the tunnel with "signals of danger" (stress molecules, aberrant expression of antigens etc.) to migrate and proliferate as they were programmed. Microglia-conditioned medium itself seems to have a beneficial influence on the survival proliferation and migration of neural precursors *in vitro* and their differentiation towards neuronal and astrocytic lineages and GBMs have notoriety to be mostly of astrocytic origin. We did not manage to find in the current literature any publication with documented transformation of neurons or terminally differentiated astrocyes (except for astrocytes when put in doses of radiation almost incompatible with life and this astrocyte-generated tumors were not even close to the biological features of GBM). Upon ischemic insult, microglia in the subventricular zone acquired a pro-neurogenic phenotype [expression of IGF-1], which is concomitant with persistent neurogenesis in the striatum after stroke. On the other hand, there has been confirmed strong correlation between factors induced by hypoxia (HIF-1alpha and beta etc.) and maintenance of large pool of constantly self renewing stem cell. In this scenario (if hypoxia is not as severe as in stroke and thus often asymptomatic), it might be logic to assume that some of these cells in state of rapid proliferation gain some potentially dangerous genetic mutations, overseen both by DNA repair mechanisms and local immune system. In fact, all conditions which are associated with increased numbers of apoptotic cells in CNS microglia acquire a phenotype that promotes adult hippocampal neurogenesis, which in first stage results in prominent proliferation of NSC who might overcome potentials of local immune system check points. It has been suggested that an increase in hippocampal neurogenesis is associated with the presence of IL-4-producing T helper 2 cells in the brain parenchyma, which could instruct resident microglia towards an IGF-1 expressing phenotype, however their number of soluble products apart IL/4 might be the "last drop" in already whirling niche.

At last, but not the least we must consider the hypothesis of inheritance of some genetic discrepancies that potentially lead to the development of GBM. However, the question is if there is an early genetic mutation why it should be privileged only for brain cells – perycytes, epiendemal cells, endothelial cells (which in proinflmmatory condition

can easily adopt antigen presenting cell properties) and microglia probably also govern it, which may lead to deep disturbances in homeostasis of whole NSC niche.

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MODERN AND ANCIENT MITOCHONDRIAL DNA GENETIC LANDSCAPE OF MATERNAL HEREDITY IN EASTERN EUROPE FROM PALEOLITHIC ONWARDS

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Mitochondrial DNA (mtDNA), a small circular genome with multiple copies per cell and maternal inheritance was found to be very useful in phylogenetic and phylogeographic studies for both modern and ancient human samples. Several characteristics recommend it as molecular tool in this type of research: it lacks introns, suffers no recombination and has a higher overall mutational rate than nuclear DNA. The segment targeted in phylogenetic and phylogeographic studies is the only non-coding region of this molecule, the control region (or D-loop, displacement loop). Mutation rate in this region is highest for the mitochondrial genome, and the mutational events affect so called mutational hot-spots. This means that mutational motifs can be discerned and grouped together in mitochondrial haplogroups that evolved from the most recent common ancestor (MRCA). Analyzing the mitochondrial human genome of both modern and ancient samples allows us to trace back migration patterns across the globe and moreover, the identification of divergence points in migration routes. Interactions between human populations through time can also be discerned, the resolution of these reconstructions being affected by biological, social and cultural aspects of the studied populations.

The multi-copy status of this genome enhances its usefulness in ancient DNA studies.

The field of mtDNA focused research in human phylogeography rapidly evolved in the last three decades. Technological advancements of sequencing capability in recent years allows rapid characterization of mutation sites relative to a universal reference sequence, the revised Cambridge Reference Sequence [1].

The matrilineal contribution to the genetic landscape of Europe is best highlighted by the characterization of the mitochondrial genome for present and past populations. An absolute association between a certain haplogroup and a geographic point of origin or archaeological culture can rarely be made. What can be characterized is the frequency of the haplogroup in a population of a certain age, occupying a known geographic range. Frequencies of mitochondrial haplogroups vary in different regions of Europe according to historical interactions of populations occupying this geographic space. Superimposing genetic data with documented geological and historical events (climatic changes, wars, pandemics etc), social and cultural characteristics (ethnicity, religion, social status, demography, population movements etc) of the populations under review may explain its genetic evolution, in a spatio-temporal frame. Most frequent mitochondrial haplogroups encountered in Europe are H, J, K, N1, T, U4, U5, V, X and W and subhaplogroups or haplotypes reunited therein. Among these clearly dominant are suprahaplogroup H and

haplogroup J. The oldest origin is attributed to haplogroup N with a tentative origin time around 75000 years before present in South Asia. Haplogroups with supposedly European and more recent origin are haplogroups X (over 30000 years ago in North-East Europe), U5a1 (30000 years ago in Europe), I (30000 yars ago in Caucasus or North-East Europe), W (25000 years ago in North-East Europe or North-West Asia), V (15000 years ago in Iberia) and H3 (10000 years ago in Spain). Rare haplogroups more frequent in Africa, Asia or the Northern American continent can be found at low frequencies. These less frequent haplogroups are usually correlated with immigration events and can often be related to specific historical events. As the speed with which human travel increased in the last two centuries, and due to relaxed interactions between distinct populations, the admixture of the European population tends to homogenize, erase, genetic signatures across the continent. In few generations, this homogenization process, the less clear population limitative factors and the demographic explosion characteristic for the civilized world will further complicate the mitochondrial genealogy of the European population, leading to a near future unsolvable genealogic tree.

The majority of current European haplogroups enter Europe at various points in time on different routes from the East. Few evolve in Europe and migrate more recently from West to North, and then East. Eastern Europe is from historical times a melting pot of diverse populations, a stop on the migration routes from the East. It was more or less isolated both from the West and the East in much of its history, and thus makes an interesting study subject in what the mitochondrial genome diversity is concerned. One of the migrations worth mentioning is associated with the import of agriculture on the European continent and the entrance in the Neolithic era. Other more recent migrations are also historically well documented and have an expected impact on the make-up of the present Eastern European population.

ACKNOWLEDGEMENTS

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THE PROBLEM OF "HEAVY METAL" POLLUTION IN ENVIRONMENT AND FISH

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Heavy metals are significant environmental pollutants, and their toxicity is a problem of increasing significance for ecological, evolutionary, nutritional and environmental reasons [1]. The term ''heavy metals'' refers to any metallic element that has a relatively high density and is toxic or poisonous even at low concentration [1], and thus are commonly defined as those having a specific density of more than 5 g/cm^3 [2]. However, no relationship can be found between density (specific gravity) and any of the various physicochemical concepts that have been used to define "heavy metals" and the toxicity or ecotoxicity attributed to "heavy metals" [3]. A classification of metals and their compounds firmly based on their chemical properties is needed, and understanding bioavailability is the key to assessment of the potential toxicity of metallic elements and their compounds [3].

The elementary constituents of plant, animal and human life may be classified as major and trace elements, the latter group comprising both essential and non-essential elements (including toxic elements) [1].

Laboratory and field observations clearly indicate that the total concentration of a metal, in solution ("dissolved" metal) or associated with particles ("particulate" metal), is rarely a good predictor of metal bioaccumulation or toxicity in aquatic organisms. Environmental scientists and managers have rather uncritically accepted the notion that some raction of the total metal concentration is "bioavailable". It has proven difficult to develop a universally applicable quantitative definition of "bioavailability", mainly because of two major factors: 1) the diversity of routes by which metals may in fact be bioaccumulated by aquatic organisms; 2) the dynamic nature of metal speciation [4].

There are two mechanisms for uptake of contaminants by biota, bioconcentration (uptake of a chemical by an organism directly from the abiotic environment) and bioaccumulation (the uptake of a chemical by an organism from the abiotic and/or biotic (food) environment, that is from all sources) [5].

Food webs depict the complex trophic interactions inherent among organisms within ecosystems [6]. Biomagnification of metals in aquatic organisms refers to an increase in tissue concentration of a given element in higher trophic levels of a specific food chain [4].

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PHYSIOLOGY AND BIOPHYSICS

ULTRASOUND - A STRESSOR THAT CAN BE APPLIED FOR GUIDANCE OF PLANT GROWTH

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Ultrasound (US) is a mechanical wave of oscillations with frequencies greater than the upper limit of the human hearing range (>20 KHz). Upper frequency limit for US is not clearly determined. It is assumed that no wave can travel longitudinally if it has a frequency higher than 5 MHz (through a gas), or 1.5 THz (through a liquid). US is applied widely as a disintegrating agent (sonication), and its lethal effect, at energies high enough, causes cell lysis due to destruction of cell structures and release of intracellular proteases. Nowadays, some attention is also being dedicated to certain beneficiary effects of low energy US, especially on plant growth and development [1,2].

Generally, the effects of US are thermal and non-thermal [3], latter being more important for the action of US in biological systems; and those could be further characterised as chemical and/or mechanical - but all relying mostly on the phenomenon of ultrasonic cavitation [4], i.e. the formation and violent collapse of small bubbles of partial vacuum (formed by the US waves travelling through a liquid medium). Cavitation in term derives local shock-waves and high propulsion in some layers of the fluid (especially layers that are next to a solid particle - situation commonly found when biological molecules exist in a watery solution).

Early works considering biological effects of low energy US on higher plants showed that such treatments could enhance plant development; it had positive effects on the growth rate, total biomass accrual, as well as the natural viability of seeds (that was negatively correlated to the thickness of the seadcoat) [3,5,6].

More recently, several hypotheses were set in order to explain the observed effects *in vivo*: increased cell membrane permeability [7] and cell wall alternations [8], increased velocity of enzymatic reactions [5,9], a hypothetical mechanism that changes the status of plant growth regulators [10]. No results are available on the possible effects on gene expression, nor are any of the mechanisms for the proposed modes of US action characterised. It remains to be elucidated, as well, what role does increased levels of oxidative stress play in the process.

Experiments on the moss *Physcomitrella patens* (Hedw.) Bruch & Schimp. are being done in order to elucidate biological mechanisms behind US-derived enhancements in plant growth, but also translate this knowledge into biotechnology. We chose *P. patens* because it is easy to manipulate and treat with US waves in an *in vitro* axenic culture, offering some exciting possibilities for characterising effects of US on several levels (ultrasctructural, physiological and epigenetic changes could be monitored), as well as prospects of using knock-out mutant lines if any genes are thought to be involved in the response process [11,12]. Other than that, there is a growing potential for using this moss as a host organism for production of a number of industrially important compounds, due to some obvious advantages of the system: cheap

cultivating conditions, easy scale-up and downstream processing, GRAS status, ability to produce complex proteins with lowered immunogenicity, possibility to make stable transgenic lines, etc. [13,14].

Preliminary results show some interesting trends [unpublished data]. US undoubtedly acts as an environmental stressor, but its effect (when dosed properly) can direct moss growth and development in a manner to rapidly produce protonemata, while gametophore growth is somewhat suppressed (when compared to control group with no treatment). More research remains to be done in order to further confirm and characterise this effect (using different approaches, as elaborated above). If sustained on a large-scale liquid culture growth conditions, these findings can be applied and implemented when growing *P. patens* for commercial usage, while molecular mechanisms shall give some important insights to plant stress response.

ACKNOWLEDGMENTS

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CALCIUM RESPONSES RELATED TO TUBULO-GLOMERULAR FEEDBACK IN RAT RENAL MICROVASCULAR MYOCYTES ARE IMPAIRED DURING DIABETES

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1. INTODUCTION

The maintaining of the renal blood flow (RBF) stability is the unique feature which is achieved by the function of number of mechanisms, divided into three groups - systemic; paracrine; autoregulatory [1]. Among number of mediators, ATP provides sympathetic and tubulo-glomerular feedback (TGF) control of the renal circulation triggering contraction of renal vascular smooth muscle cells (RVSMCs) via activation of the P2X receptors with further elevation of intracellular Ca^{2+} concentration leading to renal vasoconstriction and decrease of the RBF [2].

Diabetic nephropathies are known as one of the major factor in patients with end-stage renal failure [3]. Thus we compared the P2X receptor function in RVSMCs from control and STZ-induced diabetes.

2. EXPERIMENT

Using confocal microscopy and perforated-patch electrical recordings we explored the Ca^{2+} signalling system engaged in RVSMCs during stimulation of P2X receptors with selective agonist $\alpha\beta$-methylene ATP ($\alpha\beta$-meATP).

3. RESULTS AND DISCUSSION

Under voltage-clamp conditions 10μM $\alpha\beta$-meATP evoked P2X-mediated current similar to that induced by ATP. While there was no significant difference in the peak amplitude and time-to-peak of the P2X-mediated current in both animal groups, the time of decay to half-maximal amplitude of the current was found to be 100-150 ms longer. The dynamic of $[Ca^{2+}]_i$ changes induced by P2X receptor activation was assessed with confocal imaging of fluo-3 fluorescence. This revealed that the amplitude of the $[Ca^{2+}]_i$ transients in RVSMCs recovered within 10 min by 87% in control and only by 40% in diabetic rats while recovery of the current from desensitisation, obtained from patch-clamp experiments, was significantly slower in RVSMCs from diabetic (10-12 min) than from control (5-7 min) rats. Furthermore, the reduction of the $[Ca^{2+}]_i$ transient amplitude following block of VGCCs with 5 μM nicardipine was stronger in RVSMCs from control (by 65%) than from diabetic (by 50%) rats.

4. CONCLUSION

This results turns us to a conclusion that the mechanisms of $[Ca^{2+}]_i$ responses to P2X receptor activation in RVSMCs are altered in STZ-induced diabetes, what slows down the recovery of the responses upon repetitive stimulation of P2X receptors. This, in turn, may diminish the efficiency of TGF and sympathetic control of RBF in intraglomerular pressure regulation and thus contribute to glomerular injury in diabetes.

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QUANTIFYING AND CONTROLLING THE POWER OF PARASITES

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1. INTRODUCTION

The motility of unicellular parasites in mammals seems very interesting, yet very complex. In a world, were inertia cannot be used for propulsion, in a world of low Reynolds numbers, most of our everyday strategies of self-propulsion do not work [1,2].

One complex model organism that knows its way around is the flagellate *Trypansoma brucei*, causative agent of Chagas' Disease and Sleeping Sickness [3-9]. Trypanosomes manage not only to survive in their host's blood stream, which is a lot faster than they can propel themselves, but they even use their motility modes to get rid of their host's immune response via a hydrodynamic clearing mechanism [10,11]. Although Trypanosomes are known for more than 100 years, their motility behaviour is not completely elucidated yet.

2. EXPERIMENT

Using high-speed microscopy in combination with optical tweezers in micro-fluidic devices and analyzing the recorded data, new light has been shed on the motility of these parasites.

3. RESULTS AND DISCUSSION

Trypanosomes show elaborate motility patterns if optically trapped.

4. CONCLUSION

These results can be used to measure the power exerted by their beating flagellum. Furthermore, we designed a device to directly see and analyze the impact of any drug or chemical onto these parasites [12].

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QUANTITATIVE ANALYSIS OF TRPC4 CHANNELS GATING

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1. INTRODUCTION

Transient receptor potential channels (TRP) are non-selective cation channels, most of which are permeable to Ca^{2+}. Polymodal activation of these channels causes sodium and calcium influx and membrane depolarization in virtually all cell types. TRP channels are tetrameters with each subunit having six transmembrane domains and intracellular N- and C-termini [1]. The main aim of our research was to quantitatively characterize gating of TRPC4 channel, a member of the subfamily of TRPs, which belongs to the subgroup of receptor-activated ion channels and has prominent and well-characterised role in cholinergic excitation and contraction of gastrointestinal myocytes [2].

2. EXPERIMENT

Single channel currents were recorded in the outside-out configuration in isolated smooth muscle myocytes of the guinea pig ileum, using the patch-clamp amplifier Axopatch 200B (Molecular Devices, USA). Patch pipettes (World Precision Instruments, Inc., USA), had resistance of 3-5 MΩ when the pipette was filled with the intracellular Cs^+-based solution to block any K^+ currents. Muscarinic cation currents were activated by applying 50 μM carbachol solution. The data analysis was performed using Clampfit (pClamp 9, Molecular Devices, USA) and Origin 8.5 (OriginLab, USA).

3. RESULTS AND DISCUSSION

By constructing dwell time histograms of the closed and open states and fitting them with the exponential function using the "Automatic Compare Models" feature of Clampfit at confidence level of 0.95, we identified the presence of four exponential components for both the open and closed states of the channel. Correlation analysis showed that the channel was likely to gate between adjacent closed and open states of inversely related durations. Before jumping into a long period of long open states and short closed states, there were usually a few quick channel openings. Dwell time histogram of open state durations were fitted with 4 exponentials as shown in Fig. 1. From this we can conclude that TRPC4 has 4 different open microstates [3] with times, which on average are 0.56, 3.1, 23.8 and 109 ms, respectively. Next, using the program QuB (The State University of New York at Buffalo; http://www.qub.buffalo.edu/) and maximum likelihood method the kinetics of the transitions between the microstates channel 8 in the previously proposed general model of TRPC4 [3] has been analysed. The results of this analysis revealed the full kinetic model of TRPC4 channel gating with rate constants for all transitions as shown in Fig. 2.

4. CONCLUSION

The presence of 4 open and closed states of TRPC4 has been confirmed using the pClamp and QUB software. We have developed, for the first time, a general TRPC4 kinetics model and calculated rate constants of the transitions between its 8 open and closed microstates. The obtained rate constants show that in the 8-states model transitions between adjacent pairs of vertical states are more likely, while the horizontal transitions are less likely, explaining the nature of the cyclic activity of TRPC4. This model can be used for detailed analysis of structural and functional relations of TRPC4 and creating new pharmacological agents for the treatment of pathologies associated with enhanced visceral smooth muscle reactivity, such as IBS, COPD and OAB.

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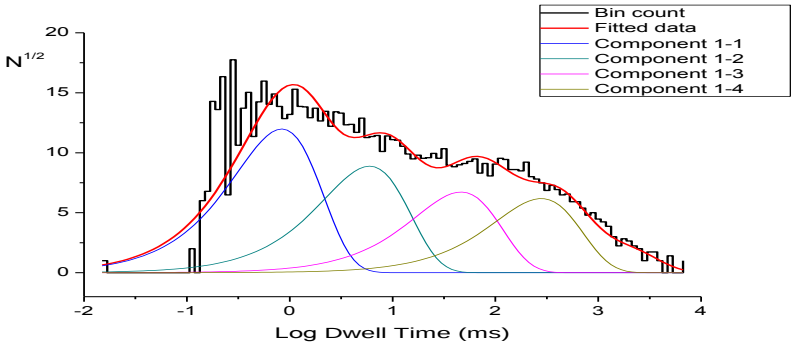

Fig. 1. *Open time histogram fitted by the sum of exponential functions with time constants and percentage contribution of each component: 1 - 0.93 (0.07), 36.1 (1.86); 2 - 9.31 (0.11), 8.26 (1.94); 3 - 118.1 (0.13), 23.5 (2.1); 4 - 964.3 (0.24), 11.55 (2.08) (standard errors are shown in parentheses).*

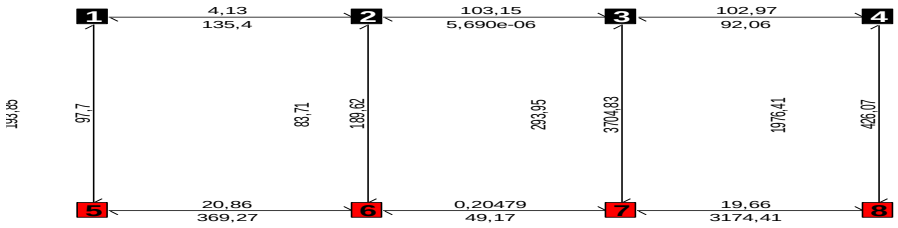

Fig. 2. *TRPC4 kinetic model of transitions between microstates with values of the rate constants (black squares marked closed state, red squares - open channel state).*

DOCOSAHEXAENOIC ACID REDUCES AMYLOID-ß INDUCED TOXICITY IN CELLS OF THE NEUROVASCULAR UNIT

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1. INTRODUCTION

Alzheimer's disease (AD) is characterized by the accumulation of amyloid-β peptides (Aβ) as perivascular deposits and senile plaques in the brain. The intake of the polyunsaturated fatty acid docosahexaenoic acid (DHA) has been associated with decreased amyloid deposition and reduced risk in AD in several epidemiological trials; however the exact underlying molecular mechanism remains to be elucidated.

2. EXPERIMENTS

The aim of the study was to test whether DHA can exert a direct protective effect on the elements of the neurovascular unit, such as neurons, glial cells, brain endothelial cells and pericytes, treated with $A\beta_{42}$ (15 µM).

3. RESULTS AND DISCUSSION

A dose-dependent high cellular toxicity was found in viability assays in all cell types and on acute hippocampal slices after treatment with $A\beta_{42}$ small oligomers prepared *in situ* from an isopeptide precursor. The cell morphology also changed dramatically. In brain endothelial cells damaged barrier function, increased para- and transcellular permeability, elevation of the production of reactive oxygen radicals, and inhibition of P-glycoprotein efflux pump was observed after peptide treatment. DHA (30 µM) significantly decreased the $A\beta_{42}$-induced toxic effects in all cell types measured by viability assays, and protected the barrier integrity and functions of brain endothelial cells.

4. CONCLUSION

These results indicate for the first time that DHA can protect not only neurons, but also the other elements of the neurovascular unit from the toxic effects of $A\beta_{42}$ and this double effect may be beneficial in AD.

5. ACKNOWLEDGEMENT

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THE IMPACT OF SPACE WEATHER ON THE BIOLOGICAL PROCESSES

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1. INTRODUCTION

This experiment allows to study the ultradian rhythmic of animals motor activity and also to find a correlation of spectrum of biorhythms with variations of ecologically factors, including factors which are controlled by space weather, to draw conclusions about the impact of natural electromagnetic fields on the biorhythms of animals.

Besides the "classic" factors such as light, temperature, partial pressure of oxygen, etc, other factors affect the biorhythms. Among these may be electromagnetic fields (EMF), which controlled by the space weather.

The experiment was conducted on white one year old rats. They were in the motion sensor wheel. Animal's motion activity was fixed during the day. Such conditions do not harm animals and allow registering motoric activity of animals.

Data on the status of space weather and geomagnetic variations were obtained from Kharkov Astrophysical Observatory (http://sw.astron.kharkov.ua/swnow.htm).

2. EXPERIMENT

Experiment was done on white mongrel rats weighing 150-200 g, females; the groups of animals were selected on their preferences in the consumption of water or alcohol. Rhythmic activity of the animals examined by automated system of animals' movement registration in a cage, allowing continuously recorded locomotor activity during the day. The animals speed was assessed in arbitrary units per second. The daily experiments were on animals that were in the wheel under normal lighting conditions and terms of deprivation of food and water. Such conditions are a little stressful and do not cause significant harm to animals, but in the integral rhythm of rats locomotor activity significantly attenuated the consumption of food and water. Mathematical analysis of time series of animals' locomotor activity was carried out based on the Fourier transform. Analysis of experimental results was made on the basis of generally accepted statistical data processing algorithms.

3. RESULTS AND DISCUSSION

The correlation and regression analysis was conducted. If we take into account the idea that periodic variations of natural electromagnetic fields cause fluctuations in the functional systems of living organisms, we have only to expect a correlation between the probabilities of detection periods in the biological and geophysical processes. The presence of positive correlation will attest to the validity of this working hypothesis, and its absence - of randomness or lack of external electromagnetic factors associated with cosmo-geophysical processes.

4. CONCLUSION

The analysis of the possible connections of biorhythms animals with variations of natural electromagnetic fields that related to space weather has been carried out separately. The rhythm of locomotor activity of animals characterized by the presence of only one fixed period – circadian, which is close to the solar day - 1440 min. Other periods are non-stationary, they can be found in different animals, or in the same, but on different days. Among the most stable periodic composite of animal activity periods, can be periods which are range 40-50 min, and also 25 and 30 min 13-16 min.

The most trustworthy period in the locomotor activity rhythm of animals corresponds highly probable geomagnetic periods, but not for the whole range of periods. The most clearly evidenced about this the correlation and regression analysis. Periods in the range 20-1,440 min. periods of physical activity to some extent synchronized variations of cosmo-geophysical factors.

Short-term biorhythms (endogenous) associated with the work of various physiological and metabolic systems of the animals are relatively stable and hardly respond to performance factors of weak geomagnetic processes. There was a more or less smooth operation of functional systems, in this range of periods (9-19 min).

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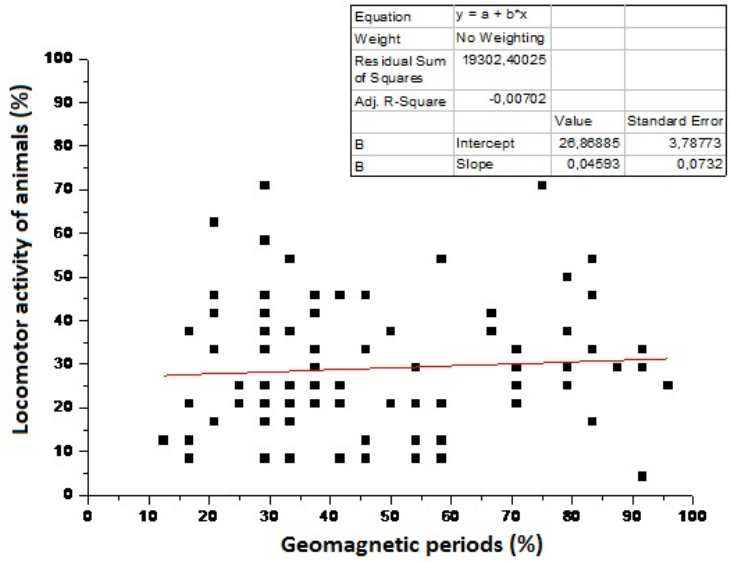

Fig. 1. *The dependence of the probability of detection periods in the rhythm of animals activity from the detection probability corresponding periods in geomagnetic data for the full range of periods.*

ULTRADIAN RHYTHMS IN LOCOMOTORS ACTIVITY OF RATS UNDER CHRONIC ALCOHOLISM

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1. INTRODUCTION

Nowadays alcohol is seen as a kind of pathological state that is characterized by addiction to alcohol. As a result of it metabolic, physiological, psychological and mental disorders are developed [1]. It is know that the various functional disorders including chronic alcoholism leads to desynchronization of biological rhythms of the body. First of all the diurnal variation of metabolic and physiological processes are disordered.

The data analysis lead us to conclude that ultradian and diurnal organization of physiological and metabolic processes in humans and animals in conditions of chronic alcohol abuse, has been insufficiently studied. The sensitivity of biorhythms to the effects of various environmental factors is also poorly understood, especially under chronic use of alcohol. Therefore, the aim of this study is to investigate violations of the structure of the ultradian and diurnal rhythm of activity in white rats, which had a long period of alcoholization.

2. EXPERIMENT

Studies were performed on white mongrel rats weighing 150 – 200 males, the groups of animals were selected on their preference in the consumption of water or alcohol. Control group includes animals that demonstrate a complete rejection of alcohol consumption. Experimental group of animals were rats preferring alcohol for water. The ethanol concentration increased during the month from 5 to 40% and thereafter remained unchanged. Animals consumed ethanol solution during their stay in cages for the content. Rhythmic activity of the animals examined by automated system of animal's movement registration in a cage, allowing continuously recorded locomotors activity during the day. The speed of the animals was assessed in arbitrary units per second. In the daily experiments, animals were in the wheel under normal lighting conditions and terms of deprivation of food and water. Such conditions are a little stressful and do not cause significant harm to animals, but in the integral rhythm of rats locomotors activity significantly attenuated the consumption of food and water. Mathematical analysis of time series of animal's locomotors activity was carried out based on the Fourier transform. Analysis of experimental results was made on the basis of generally accepted statistical data processing algorithms.

3. RESULTS AND DISCUSSION

During the first months of experimental the rats in the control group showed a weak circadian rhythm, which is probably due to the influence of the study procedure

accompanied by deprivation of food and water. At the same time in the first months of the experiment, the time organization of locomotors activity exposed to chronic alcohol abuse differed more regularity. Also there was a significant increase in the amplitude of ultradian patterns on a background of increased likelihood of daily periodicity, compared with the control group. Periods of ultradian and diurnal patterns were unstable, so the amplitude of power spectrum of the corresponding periods was small. Such changes in the temporal organization of animal's activity, probably due to simplification of the structure of biological rhythms accompanied by increased anxiety and abnormal internal synchronization of physiological processes [2]. The subsequent presence of animals in the experiment showed the restoration of the daily periodicity in the control group of rats after fifth month and the violation of it for the group of long-term alcohol consumers.

4. CONCLUSION

Summarizing our results, we received during the experiment, the following conclusions can be made:

a) Rhythm of the motor activity of animals is characterized by the presence of only one stationary period - circadian, close to solar day - 1440 minutes. Other periods are non-stationary, they are different animals, or in the same, but on different days.

b) Probably the chronic use of ethanol promotes rats phase improve the sensitivity to surrounding stimuli and increasing anxiety animals with subsequent oppression activity, accompanied by internal pathological synchronization of physiological processes in the body.

c) Amplitude ultradian periods of motor activity in rats chronically used ethanol within 4 months, significantly lower than in the controls. This suggests systemic violations of the temporal organization of the processes in the body, which is manifested as a reduction of the total number of periods and increase their sustainability.

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INVESTIGATION OF TRANSPORTER INTERACTIONS OF ANTIMALARIALS *IN VITRO*

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1. INTRODUCTION

Options to control spread of malaria are increasingly limited due to emergence of parasites resistant to widely used antimalarials, therefore, discovery of novel antimalarials appears crucial as ever. However, animal experiments are too expensive and laborious for the pharmacokinetic characterization of large number of compounds. The fate of administered drugs may largely depend on their interactions with transporter proteins, which are present in all major pharmacologically relevant barriers and in plasmodiums as well. The aim of this study was to examine whether the high-throughput cell and membrane-based transporter assays can be applied to characterize the transporter interactions of candidate antimalarials.

2. EXPERIMENT

Reference antimalarials, such as artemisinin, chloroquine, etc, have been tested for their interaction with the ABC-transporters MDR1, MRP1 and BCRP using the Solvo PredEasy ATPase kits and the interaction with the SLC family members OCT1 and OCT2 uptake transporters in cell-based assay. Measured IC50 values were correlated with the clinical observations on the tested antimalarials.

3. RESULTS AND DISCUSSION

In many case our data are the first proof for transporter interaction of these clinically important drugs. Artemisinin is a substrate for MDR1, chloroquine is inhibitor of the MDR1 and substrate for the MRP1 and BCRP, mefoquine is substrate for the MDR1 but at higher concentrations is a not specific inhibitor of all the transporters and quinine is substrate for the MDR1. These results corresponded exactly to the clinical data on the antimalarials tested.

4. CONCLUSION

We conclude that the membrane- and cell-based HTS *in vitro* assays can be applied to facilitate the ADME characterization of candidate antimalarials.

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EFFECTS OF L-ORNITHINE, INDUCER OF ACUTE PANCREATITIS IN RATS, ON CULTURED ENDOTHELIAL CELLS

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1. INTRODUCTION

A novel, non-invasive, reproducible model of severe acute pancreatitis induced by the intraperitoneal administration of basic aliphatic amino acid L-ornithine was described and characterized in rats. In our previous study on taurocholate-induced severe acute pancreatitis model elevated permeability of the blood-brain barrier (BBB) was demonstrated *in vivo*.

2. EXPERIMENT

The aim of this study was to test the direct effect of L-ornithine on viability, barrier integrity and metabolism of cultured brain endothelial cells. As an in vitro model of the BBB primary rat brain endothelial cells, pericytes and astrocytes were co-cultured using culture inserts with porous membranes.

3. RESULTS AND DISCUSSION

L-ornithine decreased the viability of brain endothelial cells at high concentrations, decreased trans-endothelial resistance and increased permeability for fluorescein and albumin across cell monolayers. Changes in endothelial cell morphology like glycocalyx continuity, tight junction, plasma and basal membrane structure and damaged mitochondria were observed in electron micrographs. L-ornithine did not elevate the early nuclear translocation of NFkB or the intracellular calcium level but increased the amount of reactive oxygen species in brain endothelial cells. Mitotracker green staining showed a disintegrated mitochondrial network in cerebral endothelial cells after 24 h treatment with L-ornithine compared to control, although no change was observed in mitochondrial membrane potential.

4. CONCLUSION

In conclusion L-ornithine treatment decreased cell viability and barrier integrity induced morphological changes in tight junctions, glycocalyx and mitochondria in cultured brain endothelial cells indicating a direct damaging effect on these cells.

5. ACKNOWLEDGEMENT

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BIOCHEMISTRY AND MOLECULAR BIOLOGY

NOVEL INSIGHT IN MECHANISMS OF ENZALUTAMIDE RESISTANCE IN PROSTATE CANCER

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Prostate cancer (PCa) is the second most commonly diagnosed cancer, as well as sixth leading cause of cancer-related death in men. Most likely to die from prostate cancer are the patients with metastatic disease, with reported PCa-specific mortality rates up to 66.1% at 10-yrs follow-up [1]. These men are classically treated with androgen-deprivation therapy (ADT), which is achieved by surgical or chemical castration using luteinizing hormone-releasing hormone agonist/antagonists. Although at first these therapies are successful, almost every patient becomes resistant to ADT, resulting in an androgen-independent cancer i.e. metastatic castration-resistant PCa (mCRPC). The latter has limited the possible treatment options, which resulted in docetaxel being the only beneficial treatment option for years. Surprisingly, CRPC remains dependent on androgen receptor (AR) functioning [2]. Mechanisms used to preserve AR signaling and mechanistic functions in CRPC are still not well studied, though several potential mechanisms have already been proposed (e.g. AR gene amplification, altered expression/mutations in co-regulators, intra-prostate synthesis of androgens, changes in kinase activation of AR, somatic missense mutations of AR, etc).

Since then, there has been an increasing interest in the development of new treatment options, directly and/or indirectly targeting the AR, resulting in a plethora of treatment strategies.

Second-generation anti-androgen enzalutamide (formerly known as MDV3100) has been approved by FDA and EMA. Enzalutamide inhibits AR translocation to the nucleus, blocks AR-DNA binding, and inhibits co-regulator recruitment. It significantly prolonged survival of men with mCRPC after chemotherapy by a median of 4.8 months in comparison to the placebo group. However, even though enzalutamide has been shown to be effective in clinical trials, most patients who initially responded eventually develop resistance.

Recent findings have proposed that enzalutamide-resistance in CRPC is supported by alternatively spliced variants of AR, NFkB pathway, F876L mutation in the AR, bypass of AR with glucocorticoid receptor (GR) or other mechanisms [3]. From a clinical standpoint AR bypass by GR is highly relevant, as patients with CRPC are sometimes co-treated with glucocorticoids. Clinical results indicate that CRPC patients receiving glucocorticoid treatment had clinical benefit; however several other studies have shown

that patients receiving glucocorticoids had worse prognosis and disease. Elucidating the role of GR in CRPC might improve treatment of PCa.
Moreover, our lab has developed enzalutamide resistant cell line (LNCaP-Rr) by culturing LNCaP cells for a long period of time in 10 µM RD162/enzalutamide. These cells show different mechanism of resistance that might be mediated by the machinery involved in cell cycle regulation.

Future research should be focused on unraveling mechanisms of resistance in patient-derived xenografts as well as conducting in-depth molecular study of already proposed mechanisms to find therapeutic targets that could be used in order to fight this advanced disease.

ACKNOWLEDGMENT

Fig. 1 was produced using Servier Medical Art (www.servier.com) for which the authors would like to acknowledge Servier.

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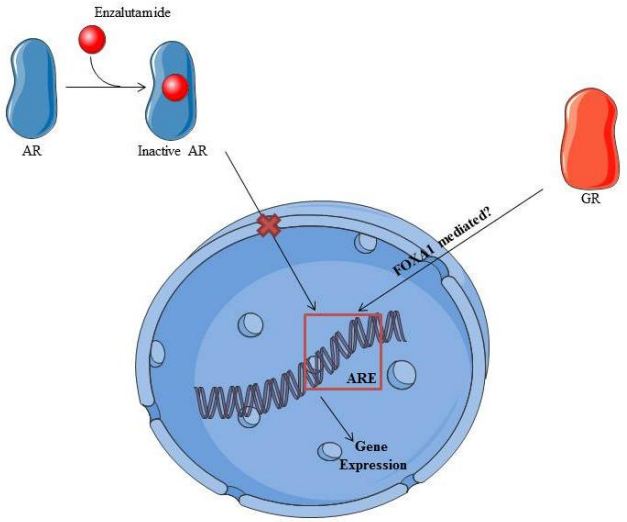

Fig. 1. *Illustration of AR bypass by GR. Forkhead box protein A1 (FOXA1) might be involved in the mechanism responsible for bypass.*

EFFECT OF BOVINE SERUM ALBUMIN ON FREE-RADICAL FRAGMENTATION OF PHOSPHOLIPIDS IN THEIR POLAR PART

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1. INTRODUCTION

Phospholipids are a major component of all cell membranes, which is why it is important to study ways of their destruction and also effective protective means.

One of the ways of lipid changes – their transformation under action of reactive oxygen species (ROS): $O_2^{\cdot -}$, H_2O_2, HClO, HO·. The major part of studies in this area deals with peroxidation of fatty acid residues in lipids leading to formation of hydroperoxides [1]. However, it has been shown recently that ROS (HO· mainly) causes a free-radical fragmentation in the polar component of the lipids containing a hydroxyl group in β-position with respect to an ester, glycoside or amide bond [2] (Scheme 1).

This process causes not only the destruction of glyco- and sphingolipids, but also the formation of glycerides, glycerophosphatides, ceramides and fatty acid amides capable of playing the role of signaling molecules in biological systems [2]. It is known [1] that a number of endogenous proteins and peptides are involved in the development and regulation of an oxidative stress in biological systems. It is not clear what kind of influence protein substances can exert on free-radical fragmentation of lipids in polar part of the bilayer.

2. EXPERIMENT

The object of study was 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (DMPG). Experiment was carried out in model membranes – liposomes. To initiate free-radical processes γ-radiation of aqueous solutions was used, and temperature was controlled with a redox-system $(Fe^{2+})Cu^{2+}/H_2O_2$/Ascorbate, at 37°C. HP-TLC and fluorescence analysis were used as methods of products determination. Phospholipids concentration was determined indirectly by inorganic phosphorus content. The changes of bovine serum albumin (BSA) occurred under γ-radiation were assessed by fluorescence analysis. Antioxidant activity of free radical scavengers was evaluated using the fluorescent probe (fluorescein 2,5 μM, pH 7,8).

3. RESULTS AND DISCUSSION

Radiation chemical yields of DMPA (Fig. 1) were: 1 - G(DMPA) = 1,24 ± 0,22 molecules/100 eV , 2 - G(DMPA) = 1,05 ± 0,09 molecules/100 eV, 3 - G(DMPA) = 0,6 ± 0,15 molecules/100 eV. It has been shown that BSA decreases the radiation chemical yield of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidic acid (DMPA) formed as result of radiation-induced fragmentation of DMPG.

Under conditions of the Cu^{2+} - mediated generation of HO· radicals (Fig. 2), BSA slowed down the rate of free-radical fragmentation in the hydrophilic part of model membrane. At the time, BSA changed its structure under the action of radiation or redox system. It was determined by fluorescence analysis.

4. CONCLUSIONS

It was concluded that albumin can inhibite free-radical fragmentation of phosphatidylglycerol by binding of Cu^{2+} iron or trapping HO· radicals. So albumin can protect components of cell membrane against free radicals, thereby showing the antioxidantproperties.

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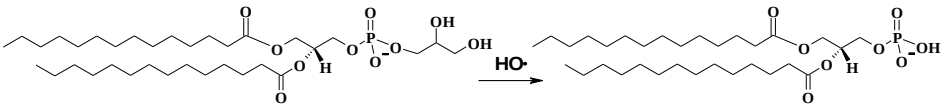

Scheme 1. *Free-radical transformation of 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DMPG) under action of HO· radicals*

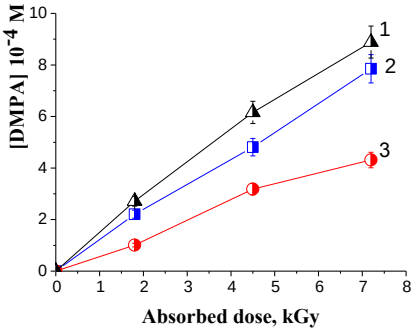

Fig. 1. *Effect produced by BSA ($2–7·10^{-5}$ M, $3–7·10^{-4}$ M) on DMPA formation in DMPG (20 mM) liposomes under γ-radiation (0,25 Gy/s).*

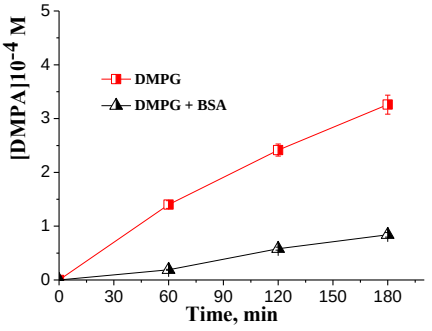

Fig. 2. *Effect produced by BSA ($1·10^{-4}$ M) on DMPA formation in DMPG (10 mM) liposomes under the action of Cu^{2+}/H2O2/Asc (0,1/1/0,1 mM) system.*

XENOBIOTIC METABOLIZING ARYLAMINE N-ACETYLTRANSFERASES IN BACTERIA AND FUNGI

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1. INTRODUCTION

Arylamine N-acetyltransferases (NATs) are enzymes involved in the detoxification of organic compounds foreign to living organisms, known as "xenobiotics". The NAT enzymes have been characterized only in mammals and in some pathogenic bacteria, but our genomic surveys [1,2] indicate the presence of *NAT* genes in many prokaryotic and eukaryotic microorganisms of potential ecological, agricultural and/or bioremediative significance. In collaboration with the USDA, we have been studying the detoxification roles of NAT enzymes in plant-pathogenic ascomycetes that compromise cereal crops and contaminate produce with carcinogenic or teratogenic mycotoxins, such as fumonisins and aflatoxins. These pathogenic fungi cause multi-million dollar losses to farmers every year and mycotoxin contamination of the food chain is considered a global health risk. Moreover, our studies suggest that the detoxification capabilities of microbial NAT enzymes, mainly of free-living biodegradative bacteria, may be useful from the perspective of environmental management and bioremediation.

2. EXPERIMENT

Thirteen *NAT* genes of 5 mycotoxigenic ascomycetes (the corn pathogen *Fusarium verticillioides*, the wheat pathogen *F. graminearum*, the tomato pathogen *F. oxysporum* f.sp. *lycopersici*, the grain contaminant *A. flavus* str. and the model ascomycete *A. nidulans*) have previously been cloned and expressed in our lab, and the enzymatic properties of the recombinant NAT enzymes have been characterized against a panel of NAT cofactors and substrates. Additionally, we have conducted extensive genomic database searches to generate a list of sequenced bacterial species with putative *NAT* genes in their genome. This *in silico* investigation supports further experimental annotation of *NAT* genes identified in phylogenetically diverse bacterial isolates from six in-house collections of our Hungarian collaborators. These collections originate from various environments, ranging from ultra pure water to highly polluted industrial sites. The *NAT* genes of biodegradative free-living *Bacilli* and various *Streptomycetes* of biotechnological utility are also investigated.

3. RESULTS AND DISCUSSION

Our work with recombinant NAT enzymes of fungi suggests the presence of two functional groups of homologues with distinct cofactor selectivity. Different NAT homologues also demonstrate diverse enzyme activities against a panel of arylamine and/or hydrazine substrates. Evidence supports that the variable detoxification capabilities of the NAT enzymes are likely to serve diverse functions within fungal cells. Comprehensive kinetic analyses and structure-function investigations are in progress, in order to understand the various enzymatic properties of fungal NATs.

Our *in silico* genomic survey for bacterial NAT homologues, has generated an exhaustive list of species and/or strains with at least one *NAT* gene in their genome. Based on this list, we have designed experimental strategies for cloning (from genomic DNA) and recombinant expression of *NAT* genes from representative species of the Hungarian collections, aiming to the subsequent enzymatic analysis of bacterial NAT proteins. Genomic DNA has been isolated from a total of 92 species and a subset of 26 species with sequenced genomes and confirmed presence of at least one *NAT* gene have been selected for further study.

4. CONCLUSION

In a world of increasing burden of water and soil environments with toxic pollutants, an in-depth understanding of microbial adaptation to natural or man-derived xenobiotics can assist global efforts for the design and application of effective bioremediation and environmental management practices.

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COMPARISON OF THREE ASSAY SYSTEMS UTILIZING A COMMON PROBE SUBSTRATE FOR STUDYING P-GP USING A SELECTED SET OF COMPOUNDS

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1. INTRODUCTION

The aim of this research was to develop a set of assays, utilizing CalceinAM as common probe substrate for the investigation of P-gp mediated drug-drug interactions.

2. EXPERIMENT

A dye efflux assay, utilizing P-gp overexpressing human cells and CalceinAM, a substrate of the transporter was used to study the inhibitory properties of the selected compound set Two membrane based assays were optimized in order to use CalceinAM, in the ATPase assay as activator molecule and as probe substrate in vesicular transport assay. The compound set, comprising 22 molecules, was chosen based on two papers (1,2), including both high- and low-permeability compounds and also P-gp interactors and non-interactors.

3. RESULTS AND DISCUSSION

Even though CalceinAM is a high permeability compound, it was successfully applied as a probe in vesicular transport studies. For the 22 compounds, IC50 values were calculated in all three assay systems. The compounds were grouped based on their passive permeability and the IC50 values compared within each group. A good correlation was observed for compounds with high passive permeability, whereas the correlation was worse for low-to-intermediate permeability compounds. The main differences were observed in the case of the dye efflux.

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ANAEROBIC FERMENTATION OF DISTILLERY THIN STILLAGE

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1. INTRODUCTION

Industrial processes create large amount of waste which are difficult and costly to decontaminate and properly dispose off. The distillery waste of starch or sugar based ethanol production is a light brown liquid with high organic content. Because of the high concentration of organics, distillery waste is a potential source of renewable energy and it can be fermented under anaerobic conditions. The main problem of the degradation of this material as a mono-substrate is the depletion of the trace elements during anaerobic degradation (AD). The distillery waste usually does not contain enough trace elements and vitamins which are necessary for the proper microbiological activity [1].

2. EXPERIMENT

The anaerobic fermentation of the distillery stillage waste was carried out in explicitly designed biogas reactors working in continuous operation mode with a working volume of 5 liters plus 1 liter headspace. The devices could be fed continuously or intermittently through a piston-type delivery system, which controls the substrate volume introduced into the digester. As the feeding was performed, the same volume of fermented material was removed through an overflow via U-shaped tubing in order to maintain a gas-tight closure and constant working volume. The biogas fermentors were equipped with a spiral strip mixing device driven by an electronic engine. In this experimental set-up we have confirmed that it is possible to degrade distillery stillage as a mono-substrate in an anaerobic reactor efficiently and continuously if the supply of the trace elements and vitamins are adequate.

3. RESULTS AND DISCUSSION

The fermentation of the distillery thin stillage as a monosubstrate was possible for 40 days (blue line). After this period the gas production rapidly decreased, from the 54. day biogas production was not observed. A possible reason for failure was the lack of the trace elements in the anaerobic reactor, which might limit the biological activity of the microbiological community. A mineral element mixture was added continuously to the parallel reactor (red line). All other operational parameters were the same and the biogas fermentation was stable at a high level until the termination of the experiment.

4. CONCLUSION

The results indicate that a stable AD fermentation of monosubstrate an adequate mineral element supply in order to sustain the viability and biological activity of the microbial community.

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PURIFICATION AND CHARACTERIZATION OF THE HTRA PROTEINASE FROM *B.SUBTILIS* 168

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1. INTRODUCTION

HtrA proteases are widely distributed in all organisms from bacteria to human [1], and are required for folding and degradation of aberrant proteins as well as for processing and maturation of native proteins [2]. We cloned the *htrA* gene from *Bacillus subtilis* into pDG148 expression shuttle-vector for overproduction of strep-tagged HtrA in *Escherichia coli*. The protein was purified on strep-tactin sepharose and partially characterized. HtrA proteinase has weaker activity compared to trypsin which is widely used in medical practice. One of the side effects of trypsin is its toxicity related to high level of proteolytic activity for both native and damaged proteins. An advantage of replacement of trypsin by HtrA could be the selectivity of its proteolytic activity for damaged proteins.

2. EXPERIMENT

Strains and plasmids: Experiments were performed with strains of *E.coli* XL-1 blue for cloning and *E.coli* BL-21 for overproduction of HtrA. Plasmid pDG148-HtrA was constructed in this work and carries coding part of the *htrA* gene with strep-tag on the C-terminus under control of SPAC promoter and *lac*-operator. The original vector pDG148 and *E.coli* strains were obtained from Prof. Dr.Karl Forschhammer (University of Tubingen, Germany).

Protein purification: The protein was purified using affinity chromatography on the strep-tactin sepharose in 5 ml column ("IBA", Germany).

Proteolytic activity assay: It was determined using chromogenic substrate azocasein (Sigma, USA). The reaction mixture (600 µl) that contained the purified enzyme and 1% azocasein in 50 mM Tris HCl/phosphate buffer was incubated for two hours under different conditions. After that, non-hydrolyzed azocasein was precipitated by 5% trichloracetic acid. Remaining hydrolysed azocasein was neutralized by 4N NaOH and the absorbance at 410 nm was measured.

3. RESULTS AND DISCUSSION

The gene of *htrA* was amplified by PCR from genomic DNA of *B.subtilis* 168 using appropriate primers and cloned into pDG148 expression shuttle-vector. A primer annealing to 3'- end of *htrA* gene had a sequence coding for Strep-affinity-tag. The recombinant plasmid was transformed into *E.coli* XL-10. The direction and sequence of the *htrA* gene was checked by PCR with appropriate primers and sequencing. The plasmid pDG148-HtrA was transformed into *E.coli* BL-21 and the overproduction of HtrA was performed. Protein overproduction was induced by 0.5 mM isopropylthiogalactoside (IPTG) using cell culture with OD_{590}=0.7. Four hours after

induction the cells were harvested and lysed by sonication in the StrepWash buffer (150 mM NaCl, 1 mM EDTA, 100 mM Tris-Cl, pH 8.0). Crude cell lysate was mixed with Strep-Tactin and loaded into 2-ml column. HtrA-ST was eluted by desthiobiotin containing buffer (150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin, 100 mM tris-Cl, pH 8.0). The protein purification quality was checked by the SDS-PAGE.

Physicochemical properties of HtrA were studied using hydrolysis of azocasein by HtrA protease under different temperature and pH. All manipulations were made as described above. Maximal activity was observed at 32°C and pH 7,0 (Tris HCl – buffer conditions) (Fig. 1).

4. CONCLUSION

Obtained data are not consistent with literature data [3] and expected results. This may be related to the enzyme modification by Strep-tag. On the other hand acquired data of HtrA activity are lower in compare with trypsin that is used nowadays as vulnerary medication. Also trypsin that is the most preferred proteinase in medical practice has optimum of temperature 50°C and pH 8.5. Taking into account that pH of obtained proteinase is close to pH of skin and blood of human it is possible to expect minimal changes of its properties in therapeutic use.

5. ACKNOWLEDGMENT

This research was supported by RFBR grant 12-04-31472 and by Ministry of Education and Science of the Russian Federation.

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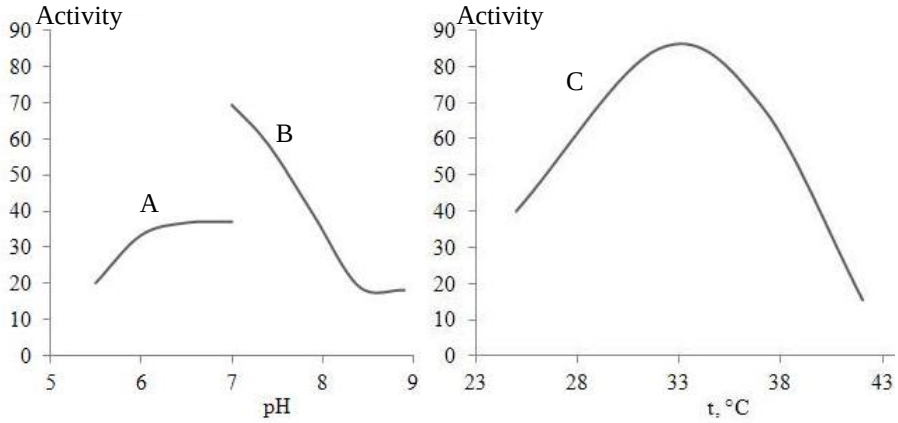

Fig. 1. *Proteolitic activity of the HtrA proteinase. A – phosphate buffer, t=37°C. B – Tris HCl buffer, t=37°C. C – under different temperatures, pH=7.0.*

THE ROLE OF NUCLEAR LAMINS IN NUCLEAR ORGANIZATION AND CELLULAR SIGNALING

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1. INTRODUCTION

Lamins are the major architectural proteins of the nucleus and are important for nuclear integrity and assembly. They are involved in the organization of nuclear functions. Lamin genes mutations lead to highly debilitating genetic diseases that affect many different tissues such as muscle, adipose and neuronal tissues, or cause premature aging syndromes. There are two types of lamins – A and B type. In mammals, two major A-type lamins (lamin A and C) and two major B-type lamins (lamin B1 and B2) have been characterized. In addition, there is ultrastructural evidence proposing that lamins are part of a nucleoskeleton distributed in the nucleoplasm. The observed interactions of lamins with inner nuclear membrane proteins, chromatin, and various regulatory factors have given information regarding the role of lamins in cellular processes and tissue-specific signaling pathways.

2. EXPERIMENT

a) Analysis of HeLa nuclear lamina preparations.
b) UV cross-linking experiments.
c) Fluorescence *in situ* hybridization (FISH) microscopy.

3. RESULTS AND DISCUSSION

Over the past few years research has focused a lot in the nuclear lamins. Although it is known that the positioning of interphase chromosomes inside the nucleus is non-random, it remains unclear how this spatial organization is created and what molecules are involved. One possible mechanism suggests that nuclear lamina has a critical role. Nuclear lamina is a threadlike protein layer that comprises A- and B-type lamins and coats the nucleoplasmic side of the inner nuclear membrane. Moreover it interacts with hundreds of large genomic regions termed lamina associated domains. Mapping has revealed that lamina associated domains can be found in all mammalian chromosomes and their genomes contain about 1,100–1,400 of them, that specifically associate with the nuclear lamins. This proportion suggests that lamina associated domains-nuclear lamina interactions impose major constraints on the shape and positioning of chromosomes. Fluorescence *in situ* hybridization (FISH) microscopy of numerous lamina associated domains has indicated that they are indeed preferentially, but not always, located near the nuclear lamina. This suggests that lamina associated domains-nuclear lamina interactions are also dynamic. However, FISH can only be performed in fixed cells, and due to the limited resolution of light microscopy it is impossible to know whether a lamina associated domain makes direct molecular contact with the nuclear

lamina, or is merely nearby. Studies of normal cells and cells from the multitude of laminopathy patients show that these proteins play important roles in various nuclear functions, including transcription, DNA replication, DNA repair, as well as cell proliferation and differentiation. However, the detailed mechanisms remain unclear. In addition, lamins are thought to take part in the structural organization and epigenetic regulation of chromatin. Although it is clear that chromatin organization and histone methylation are changed in diseases caused by mutant lamins, virtually nothing is known about the specific functions of lamins in these processes under normal physiological conditions. Lamins may be involved in the regulation of epigenetic changes responsible for cell differentiation, as they are expressed in a developmentally regulated manner. In addition, different disease-causing mutations appear to differ in their structural properties *in vitro* and *in situ*, suggesting that they affect the various lamin structures present in the nucleus in different ways.

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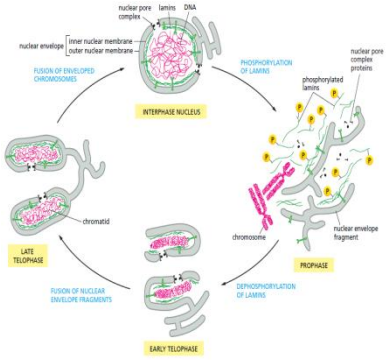

Fig. 1. *Breakdown and re-formation of nuclear envelope during mitosis. Phosphorylation of the lamins starts the disassembly of the nuclear lamina, which helps the nuclear envelope to break up. Dephosphorylation of the lamins helps to reverse the process.*

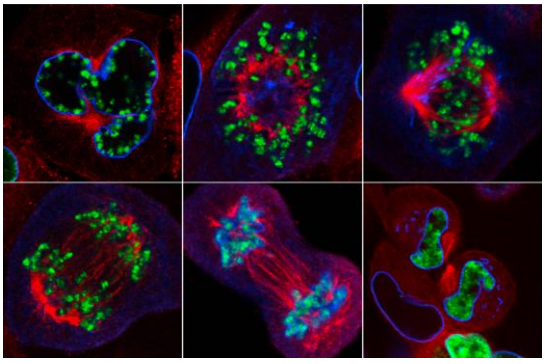

Fig. 2. *Lamin Associated Domains distribution at different stages of mitosis. Confocal sections show m6A-Tracer (green), Lamin B1 (blue), and alpha-tubulin (red).*

DETERMINATION OF ISOTOPIC FRACTIONATION BETWEEN FREE Zn AND Zn-EDTA AND ITS IMPLICATION FOR Zn UPTAKE IN PLANTS

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1. INTRODUCTION

Food sources rich in Zn are vital for human health as various biochemical pathways are controlled by this trace element [1]. Laboratory studies have revealed that graminaceous plants can increase Zn uptake by secretion of phytosiderophores (PS) from the mugineic acid family of amino acids (MA). These are very important metal chelators, which bind free Zn^{2+} in soils deprived from Zn and transport the stable complex to the cell transporter protein increasing its solubility [2].

The importance and role of Zn-PS complexes in plant growth are difficult to study *in situ* due to the complex and numerous processes occurring at the soil-plant interface. The use of stable isotopes, however, has shown a great promise in disclosing the mechanisms that take place in soil-plant environment. [3-5]. In this presentation, we demonstrate that the formation of organometallic complexes has a tendency to introduce a heavy isotopic enrichment in the environment.

2. EXPERIMENT

Synthesis of high-purity MAs is cumbersome [6], therefore a synthetic ligand with similar binding properties was chosen as a model ligand - ethylenediamine tetraacetic acid (EDTA).

To design appropriate reaction solutions at the required conditions for this experiment GEOCHEM-EZ software was used. The speciation was calculated in order to achieve complete complexation with Zn: for equimolar solutions of $Zn(OAc)_2$ and EDTA (pH 6.3) the calculations forecasted that 99.98% free Zn^{2+} would complex with EDTA ligand. Consequently, different fractions of free Zn^{2+} to total Zn^{2+} were prepared (0, 0.5, and 1 mole fraction).

The separation of Zn-EDTA complex from free Zn^{2+} was achieved using an ion exchange chromatographic technique that uses Chelex-100 resin (sodium form, 100 mesh) (procedure modified from [7]). The ICP-AES spectrometer was used to determine Zn concentration in samples, whereas Zn isotope ratio measurements were carried out using ICP-MS. To avoid possible interferences of Na with Zn isotope ratio measurements, the solutions after the Chelex 100 passage were submitted to an anion exchange separation procedure.

3. RESULTS AND DISSCUSION

Fig. 1 (b) and (c) show that Zn-EDTA complex was eluted instantly with buffer solution (0.5M NaOAc), whereas the free Zn^{2+}, initially exchanged with the resin, was eluted upon addition of 1M HCl. Thus, the chelating resin (Chelex-100) was successfully applied for the separation of free Zn^{2+} from Zn-$EDTA^{2-}$ at pH 6.3.

The value of $d^{66}Zn_{(Zn2+)}$of the 5 fractions ranged between -0.19 and 0.16, for fractions 0 and 1. The consistency of the determined Δ and the linear relationship (data not shown) suggests equilibrium. This enables us to calculate the equilibrium fractionation to 0.29 + 0.09 ‰. Consequently, our results show that the isotopically heavy Zn is preferentially incorporated into the Zn-EDTA complex.

4. CONCLUSION

By forming covalent bonds with Zn, organic ligands, such as PS, favour heavier isotopes, therefore inducing positive fractionation values. The findings from this study provide further support, that the metal complexation by organic ligands, similar to PS, is a mass biased process, which can induce a change in isotopic variability in biogeosystems [3-5,8]**.**

The measured results support the hypothesis that fractionation is induced by the formation of the complex. The Zn isotope fractionation, presented here, supports the interpretations from both field [3] and laboratory studies [4] that Zn efficiency is linked to uptake of Zn-ligand complexes.

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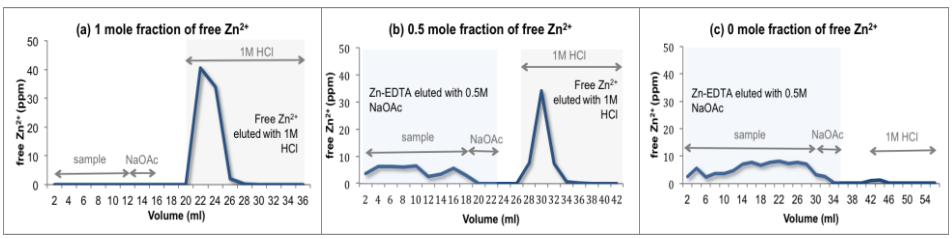

Fig. 1. *Elution profile of* ***(a)*** *1 mole fraction of free $Zn^{2+}$to total Zn (free Zn^{2+})* ***(b)*** *0.5 mole fraction of free $Zn^{2+}$to total Zn, with 0.5 M NaOAc (Zn-$EDTA^{2-}$) and 1M HCl (free Zn^{2})* ***(c)*** *0 mole fraction of free $Zn^{2+}$to total Zn (Zn-$EDTA^{2-}$). Total Zn loaded in each case was 0.65mg, whereas the average recovery was 93.8%).*

IMMUNOLOGY AND MICROBIOLOGY

GENOTYPES, SUSCEPTIBILITY PROFILE AND VIRULENCE OF *CRYPTOCOCCUS NEOFORMANS* CLINICAL ISOLATES FROM SERBIA

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1. INTRODUCTION

Cryptococcus neoformans causes systemic fungal infection mainly in individuals with a suppressed immune system. Recent study estimated that annually approximately one million HIV-infected subjects develop cryptococcal meningitis of which circa 625,000 will not survive this disease [1]. Molecular and biochemical tools revealed that this species consists of varieties: *C. neoformans* var. *grubii* (serotype A; genotypes AFLP1, AFLP1A and AFLP1B) and *C. neoformans* var. *neoformans* (serotype D; genotype AFLP2), as well as hybrids between both cryptococcal varieties (serotype AD; genotype AFLP3) [2]. Also, each *C. neoformans* strain can exist in form of haploid MAT**a** or MATα cell. MATα strains have been shown to be much more common and more virulent. Besides the presence of MATα locus, capsule formation and the production of melanin and phospholipase have been identified as virulence factors of *C. neoformans* strain. Studies of cryptococcosis in animal models infected with different strains of *C. neoformans* have indicated that there is considerable variation in the virulence, susceptibility and ecological characteristics of individual isolates.

Molecular epidemiological surveys on the genetic variety of cryptococcal isolates have been described for various European countries, but for Eastern Europe data is missing. The purposes of this study were: (i) to identify the genotypes, serotypes and mating types; (ii) to determine antifungal susceptibility profile; (iii) to analyze virulence factors of *C. neoformans* isolates.

2. EXPERIMENT

This study investigated 34 clinical Serbian *Cryptococcus neoformans* isolates from 25 patients in the 20-year period. Amplified fragment length polymorphism (AFLP) fingerprinting was used for genotyping, while real-time PCR was used to determine the mating- and serotype. The MICRONAUT plates were used to determine susceptibility to amphotericin B, 5-fluorocytosine, fluconazole, itraconazole, posaconazole and voriconazole, according to the CLSI standardized protocol. Each strain was examined for production of: (i) capsule by India ink staining; (ii) melanin by culturing of minimal L-DOPA medium and (iii) extracellular phospholipase by culturing on Sabouraud-egg yolk agar.

3. RESULTS AND DISCUSSION

AFLP analysis revealed that the majority of isolates belonged to genotype AFLP1 (58.8%), followed by AFLP2 (29.4%), AFLP3 (8.8%) and AFLP1B (2.9%). Out of 25 patients, 21 (84%) were infected with MATα isolates. This genotype and mating type distributions are consistent with previous European studies [3-5].

In this study, all isolates were found to be susceptible to amphotericin B, posaconazole and voriconazole, as previously shown [3-5]. Comparing to similar studies, the percentage of isolates resistant to fluconazole (2.9%) was lower [4, 6], while the percentage of 5-fluorocytosine resistant isolates in this study (5.9%) was higher [4, 5]. A high percentage of isolates was found to be susceptible dose-dependent to itraconazole (47.1%), similar to previously reported [4,5].

In relation to underlying disease, the patient's gender, as well as mating-, sero- and genotype of the *C. neoformans* isolates, analysis showed that there was only one statistically significant difference, namely that isolates obtained from HIV-negative individuals are significantly less susceptible to 5-fluorocytosine (*t* test, $p<0.01$). Having in mind small number of HIV-negative patients, these results should be interpreted with caution. All clinical isolates produced phospholipase, capsule and melanin, but these activities varied with individual isolates. In relation to production of virulence factors, our results showed that AFLP2 isolates produced more melanin, comparing to AFLP1 and AFLP3 (*t* test, $p<0.05$). Also, within AIDS group of isolates, positive correlation between production of melanin and phospholipase was shown (Pearson's correlation, $r=0.5$, $p<0.05$).

4. CONCLUSION

This is the first epidemiological study of genotypes, virulence and antifungal susceptibility of *C. neoformans* clinical strains isolated in Serbia.

5. ACKNOWLEDGMENT

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GENERATION OF OVER-EXPRESSION AND KNOCK-OUT LIBRARY IN THE HUMAN PATHOGENIC YEAST *CANDIDA PARAPSILOSIS*

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1. INTRODUCTION

Over the recent years there has been exponential growth in the number of yeast genome sequences. Despite the growth of sequence information, a large number of fungal genes remain uncharacterized and the functions of genes are based on sequence homology. There are two main approaches for studying gene function: one is to generate strains that over-express the gene of interest under the control of a strong promoter and the other is to generate knock out mutant strains. Previously over-expression strategies have been applied to investigate *Candida albicans* gene function, interaction with the host and virulence attributes [1,2]. In our previous work several fungal transcriptional factors have been identified using RNA-Seq data, which were over-expressed during host-pathogen interactions. Based on these data and using the Gateway™ technology we were able to generate *C. parapsilosis* strains that over-express our genes of interest. For this, *C. parapsilosis* CLIB 214 *leu-* strain was used. Using the *caSAT1* flipper system we have integrated the *RP10* locus of *C. albicans* SC5314 to the *RP10* locus of *C. parapsilosis* CLIB 214 *leu-* strain. With this integration we were able to adopt the *TDH3p-CLP10* over-expression system established in *C. albicans*.

2. EXPERIMENT

A *TDH3p-CLP10-GFP* construct was used to test whether this system is able to express the genes of interest in *C. parapsilosis*. For entry vectors the pDONR 207 was used, while for destination vectors the *TDH3p-CLP10* containing vectors were applied. All of the transformants were bar-coded using a 20 bp tag.

For knock-out mutant library the *C. parapsilosis* CLIB *leu-*/*his-* strain was used. Fusion PCR method was applied to generate specific deletion constructions. Primarily we generated the flanking PCR products for the upstream (5'-flanking) and downstream (3'-flanking) regions for each of the genes, and the HIS1 and LEU2 marker PCR products. We used HIS1 marker from plasmid vector pSN52, and LEU2 from plasmid vector pSN40 [3]. For each of the identifications we used colony PCR to confirm the total deletion of the genes. All of the mutants were bar-coded using a 20bp tag in order to be able to identify them during later *in vivo* infections.

3. RESULTS AND DISCUSSION

The null mutant strains are continuously being tested in different conditions such as growth abilities on certain temperatures and on different medias observing pseudohyphae formation as well. So far we have found null mutants that show

differences in appearance such as increased pseudohyphae forming or regressed growth on different temperatures.

4. CONCLUSIONS

The comparison of the virulence of these null mutant strains with the use of infectious models is now in progress.

In the future with the use of this method we are able to identify key regulatory factors that may play a role in the virulence of *C. parapsilosis* during host-pathogen interactions.

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UNUSUAL BEHAVIOR OF *CANDIDA PARAPSILOSIS cdr1-2* DOUBLE DELETION MUTANT AGAINST IMMUNE CELLS

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1. INTRODUCTION

The incidence of invasive fungal infections has significantly increased over the past 30 years, with the *Candida* genus representing the most common cause of disease. *Candida parapsilosis* is now the second or third most common cause of bloodstream infections in intensive care units. Triazoles are commonly used to combat diverse forms of candidiasis. The prolonged and frequent use of azoles drugs has led to the development of multidrug resistance (MDR) mechanisms in *Candida*. Several mechanisms have been characterized that contribute to MDR in these yeasts. One of these mechanisms is the overexpression of efflux pump proteins that function as ATP-binding cassette (ABC) and major facilitator super-family (MFS) transporters. Major *C. albicans* ABC-transporters involved in azole resistance are candida drug resistance protein 1 and 2 (Cdr1p, Cdr2p).

2. EXPERIMENT

In contrast of the well studied *C. albicans* ABC-transporters, little is known about the role of *CDR* genes in *Candida parapsilosis*. To clarify the significance of CpCDR, we have generated *C. parapsilosis CDR1-CDR2* (*CpCDR1-CDR2*) double deletion mutant and determined the minimal inhibitory concentration (MIC) for amphotericin B, fluconazole and caspofungin.

3. RESULTS AND DISCUSSION

Our results showed decreased MIC for fluconazole and caspofungin, respectively. Flow cytometry experiments using calcein dyeing was performed to determine the structure specificity of Cdr1p and Cdr2p beside the ABC-transporter super-family. Our data demonstrated that the double deletion mutants were stained on a higher level than the wild type, respectively. This staining feature is typical to a mammal ABC-transporter called MDR1. The knock out mutants showed similar sensitivity against osmotic and oxidative stress to that of the wild type. Interestingly, the killing of *CDR1-2* deletion mutant yeasts by murine monocyte-like cells (J774.2) was significantly decreased relative to WT. Furthermore, we showed that *CDR1-2* deletion yeast cells were more resistant against the host killing mechanisms during infection of A/J mice, suggesting an involvement of these transporters in the pathobiology of the fungus.

4. AKNOWLEDGEMENTS

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EVALUATION OF SOME GLIOMA STEM-RELATED MARKERS IN ORIMARY HIGH-GRADE GLIOMAS

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1. INTRODUCTION

Glioblastoma multiforme (GBM) is the most common primary tumor of the brain, as well as one of the deadliest tumors, persistant to all forms of threatment.Both classical pathology and recent WHO classification assign so called primary GBM or "classical subtype", which develops without any symptoms or diseases and has the worst prognosis. In the last decades, the treatment strategies for GBMs have not changed appreciably. The most rapidly developing field within cancer research is recently focused on "tumor stem cell" hypothesis. According to this hypothesis GBM stem cell (GBMSC) could be defined as malignant cell subpopulation that possess characteristics associated with normal stem cells. Conventional therapies seem to shrink bulk tumor mass, but bypass GBMSCs which therefore easily reestablish GBM. There is a clear need for reevaluation of GBMSC-related markers in large cohorts of patients and retrograde large-scale tumor samples analysis. The aim of this study was to investigate expression of few proposed GBM-stem related markers: CD133, Sox-2, Msi1&2, Nanog and β-catenin.

2. EXPERIMENT

This retrospective study employed the most representative pathohistological samples from 117 consecutive patients with primary GBM, before they had undergone surgical removal, radio- chemo- or adjuvant therapy. Results were quantified using Cell Profiler, on 5 fields with minimum 1000 cells per field.

3. RESULTS AND DISCUSSION

CD133 ICH staining showed fields with diffuse expression, but CD133+cell clusters could be also observed in close proximity to the glomerules. Mostly fibrillary tumor cells showed positive processes and a perinuclear rim devoid of staining (Fig. 1a) and significantly correlated with survival of GBM patients. Msi-1 (Fig. 1b) shoved clear intense cytoplasm staining and the highest correlaton with both survival and the time of relapse ($p<0.0001$). Msi-2 showed no characteristic pattern of expression, or connection with clinical data.Sox-2 staining was positive predominantly in two pathohistological cellular types: the small undifferentiated cells and cells with large pleomorf nuclei. Cells were predominantly clustered around small blood vessels with significant angioprolifertation, which may suggest localization of GBMSCs niche (Fig. 1c). Moreover, since CD133 and Msi-1 were was found on the surface of tumor cells in

invasive front, this relation may have a potential as clinical marker in patients suffering lower grade gliomas. There was no correlation between the Sox-2 and Nanog expression. Moreover, Nanog expression levels were variable, but Nanog may be interesting as a genetic subtype marker in this cohort of patients and might show to be another pathogenic important point in the development of certain high grade gliomas. Tumors that expressed Nanog were very aggressive.EGF signaling results in the phosphorylation of α-catenin and promotes β-catenin transactivation, which correlated with our results which showed high levels of its expression.

4. CONCLUSION

Establishing panel of sensitive molecular markers essential for survival and self-renewal of GBM will allow for selective therapeutic targeting. From this large-scale retrospective analysis we suggest β-catenin, Msi-1 and Nanog (if expressed) as promising molecular targets for primary GBM treatment.

5. ACKNOWLEDGMENT

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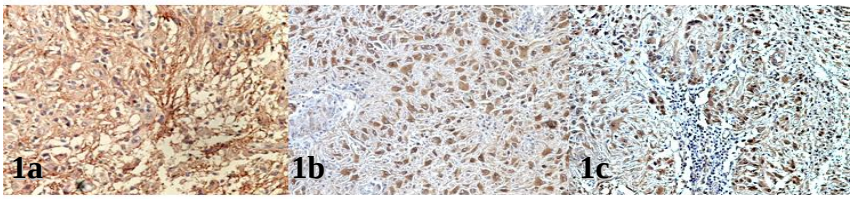
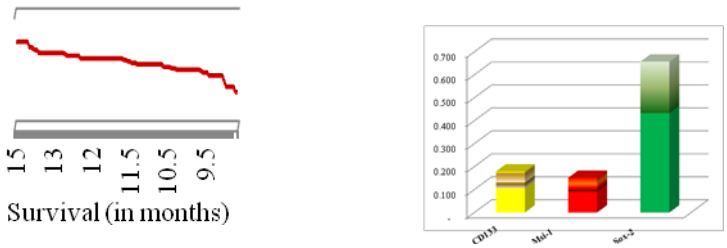


Fig. 1. *GBM ICH staining for a) CD133; b) Msi-1; c) Sox-2.*



Graph 1. *Negative correlation between survival and stem-related markers.*

ISOLATION AND PURIFICATION OF A NEW BACILLARY PHYTASE

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1. INTRODUCTION

Phytates account for 50% of the total organic phosphorus in the soil and more than 80% of the total phosphorus in forage. The phytase enzymes hydrolyze phytates to form inositol and accessible phosphoric acid salts. Microbial phytases capable of degrading insoluble soil phytates possess a high practical potential and can be used as soil fertilizers and additives to forage [1]. Phytases catalyze the successive hydrolysis of phytates into less phosphorylated inositol derivatives, accompanied by the release of inorganic phosphate [2]. After the addition of phytases, there is no need for the exogenous administration of phosphorus, which beneficially affects the environment [1]. It has been experimentally established that microbial phytases, especially in symbiotic plants and animals, play a key role in the mineralization of organic phosphorus.

The aim of work was to isolate and purify the *Bacillus ginsengihumi* β-propeller phytase from the cells of a recombinant strain, as well as to determine the structure and some properties of the protein.

2. EXPERIMENT

B. ginsengihumi phytase was isolated from the cells of the recombinant *Escherichia coli* strain Rosetta *pET-LIC/Phy* by affinity chromatography on Ni-granules, ion-exchange chromatography on Q-Sepharose, and gel filtration on Sephadex G200 column in a system of rapid-resolution liquid chromatography. The homogeneity of the purified enzyme was also confirmed by electrophoresis.

The primary structure of the purified *B. ginsengihumi* phytase was determined by MALDI-TOF mass spectrometry.

3. RESULTS AND DISCUSSION

After three stages of the purification of cell lysates, we obtained a phytase preparation with a purification degree of 500 and a yield of activity of 9.1%. The method of affinity chromatography made it possible to obtain a chromatographically homogeneous phytase preparation from the lysates of the recombinant strain. The homogeneity of the purified enzyme was also confirmed by electrophoresis; only one protein band was detected in 12% PAG in the presence of SDS. The molecular weight of the protein was 41 kDa. It was found that the amino acid sequence of phytase is identical to that obtained from the nucleotide sequence of the *B. ginsengihumi* phytase gene we sequenced. The isoelectric point of *B. ginsengihumi* phytase, which was determined based on the structure, was pI=4.8.

4. CONCLUSION

The amino acid sequence of phytase contained 371 amino acid residues, which corresponded to the molecular weight of 41 kDa and confirmed the data obtained by SDS electrophoresis.

5. AKNOWLEDGMENT

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ANTIFUNGAL ACTIVITY OF TEN BIOCIDES AGAINST MOLDS ISOLATED FROM TWO ROMANIAN CHURCH FRESCOES

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1. INTRODUCTION

In the last few years, studies carried out in Romania revealed that fungal attacks occur in improper maintenance conditions of churches such as intermittent heating, high humidity or inadequate substances used in restoration. Currently, scientists use several methods of treatment like chemical, physical, mechanical or biological, but most of these treatments attack the mineral substrate, pollutes the environment, being effective only on short term. A modern solution to reduce the bio-deterioration is to use biocides based on essential oils as an environmental alternative. In this paper, the antifungal effect of ten biocides was investigated against moulds isolated from mural paintings of two churches from Buzău County: Băbeni and Drăgheşti.

2. EXPERIMENT

Fungi were isolated from the frescoes. A volume of 0.5 ml of spore suspension from each sample was added in Petri dishes and MMA medium was pored over. Small round paper discs were impregnated with 5µl of each biocide and distributed directly on the Petri dishes. Ten biocides: Biotin T (B), P6, Algophase (AL), C3, Cinnamaldehyde (CI) , C2, P2, Preventol (P) , New Des (ND) and Capsaicin (CS) were diluted in sterile distilled water, and for the experiment concentrations of 3% and 5% were used. C2 and P2 are based on essential oils and are still tested and AL was in use for the first time on frescoes. IA of biocide was determined using the diffusion method and values of MIC were compared to establish the remanence of each biocide.

3. RESULTS AND DISCUSSION

After two weeks, the effect of all biocides was: B>C3>AL>CI>> C2>P>ND>P6>P2>CS for the both studied frescoes. In the case of Drăgheşti church, C3 was much stronger then Biotin T at 5% concentration. The IA was monitored for four months and their remanence was: P>C2>ND>B>P2.

4. CONCLUSIONS

Results indicated that the antifungal activity of biocides is different, depending on moulds type and concentration of each used substance. In the case of Băbeni moulds, a yellow pigmentation appear in all samples; this phenomenon is not present in Drăgheşti moulds, but here a strong pink pigmentation appear due to species of the *Fusarium* genus. After two weeks of action, the power of all biocides is: B>C3>AL>CI>>C2>P>ND>P6>P2>CS for both studied frescoes. In the case of Drăgheşti church, C3 was much stronger then Biotin T biocide. The IA was monitored for a period of four months to determine the effect of biocides used *in vitro*. After this period, the action power of biocides who kept their remanence is: P>C2>ND>B>P2. Preventol is the only biocide whose remanence is constant modified regardless the type of tested mould; also C2 have a strong remanence in both studied cases.

In conclusion, it is essential to identify all the genus of fungi species from the frescoes before restoration and, if possible, apply a specific treatment for each species to identify the best solution to stop this type of attack.

5. ACKNOWLEDGEMENTS

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ANTI-*ASPERGILLUS* ACTIVITY OF *ORIGANUM VULGARE* L. ESSENTIAL OIL

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1. INTRODUCTION

Oregano (*Origanum vulgare* L., Lamiaceae) is widely known as a plant which has been used in agricultural, pharmaceutical and cosmetic industries [1]. Essential oil of this plant is recognized as an excellent natural source of substances with antimicrobial activities such as terpens and phenolic compounds [2]. *Aspergillus* is a genus of fungi, including over 180 species, common contaminants of various substrates [3]. Several species have attracted attention as human and animal pathogens or because of their ability to produce mycotoxins. Therefore, the aim of this study was to evaluate the antifungal activity of *O. vulgare* essential oil on selected *Aspergillus* species.

2. EXPERIMENT

Eight *Aspergillus* species were isolated from different supstrates: *A. flavus, A. parasiticus, A. ochraceus, A. niger, A. terreus, A. fumigatus, A. nidulans* and *A. chevalieri*. 10 µl of spore suspension from each sample was added into wells of a microdilution plate (96 well-plate) containing MEB medium. *O. vulgare* essential oil was added in different concentrations (0.1 µl/ml - 5 µl/ml) and microdilution plates were incubated for 7 days at 25°C. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) were determined and compared between the isolates.

3. RESULTS AND DISCUSSIONS

After the incubation period, MIC value for essential oil ranged between 0.25 µl/ml and 2.5 µl/ml, and MFC value varied between 1 µl/ml and >5 µl/ml (Tab. 1). The lowest MIC value (0.1 µl/ml) was found at interactions of essential oil with *A. chevalieri* and the highest (2.5 µl/ml) with *A. terreus*. On the other hand, the lowest MFC values (1 µl/ml) were founded with *A. flavus, A. parasiticus* and *A. chevalieri* and the highest (>5 µl/ml) with *A. niger* and *A. terreus*.

The results of the microdilution method showed that various concentrations of *O. vulgare* essential oil exhibited both fungistatic and fungicidal activity on tested *Aspergillus* strains. Relatively strong antifungal activity of *O. vulgare* essential oil can be attributed to high concentrations of some phenolic compounds (mostly carvacrol and thymol) in its content [1,4,5]. Phenolic compounds, at appropriate concentrations, are reported to be effective against some microorganisms [1]. Their mechanism of antimicrobial activity is related to disruption of microbial cell wall and precipitation of cell proteins [1]. It is suggested that the presence of an aromatic nucleus with OH group is responsible for making hydrogen bonds with active sites of target enzymes [1,6].

Therefore, it is possible to suppose that these groups are responsible for antimicrobial activity.

Tab. 1. MIC and MFC values of *O. vulgare* essential oil on tested *Aspergillus* species.

Investigated species	MIC [µl/ml]	MFC [µl/ml]
A. flavus	0.5	1
A. parasiticus	0.5	1
A. ochraceus	1	2.5
A. niger	0.5	>5
A. terreus	2.5	>5
A. fumigatus	0.5	2.5
A. nidulans	0.5	2.5
A. chevalieri	0.25	1

4. CONCLUSIONS

The essential oil of *O. vulgare* exhibited significant antifungal activity on eight tested *Aspergillus* strains. The lowest MIC value was found at interactions of essential oil with *A. chevalieri* and the highest with *A. terreus*. The lowest MFC values were found with *A. flavus*, *A. parasiticus* and *A. chevalieri* and the highest with *A. niger* and *A. terreus*. Significant antifungal activity of *O. vulgare* essential oil is attributed to high concentrations of some phenolic compounds in its content, mostly carvacrol and thymol.

5. ACKNOWLEDGEMENT

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BIOLOGICAL ANTHROPOLOGY

MOLECULAR DIAGNOSIS OF A TUBERCULOSIS CASE FROM THE LATE-ROMAN PERIOD

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1. INTRODUCTION

Worldwide, 2 million people die every year of tuberculosis. In humans, this disease is caused by several species which belong to the *Mycobacterium tuberculosis* complex. Today, it is easy to diagnose modern cases of tuberculosis, and even identification of *Mycobacterium tuberculosis* strains, but this task is difficult when we have to deal with archaeological human skeletal remains. Bone lesions appear in 3-5% of tuberculosis cases and those can be used to diagnose the disease in ancient skeletal remains [1].

Ancient cases of tuberculosis cannot be diagnosed using only the morphological signs and the disease should be confirmed by molecular methods. This research is focused on distinguishing between *Mycobacterium* species and strains using a set of specific SNPs in the bacterial DNA [2]. For this purpose we use human remains from the 4^{th}-5^{th} century AD that display symptoms of tuberculosis resulting in Pott's disease. In order to achieve this goal, we analyze two categories of biomarkers: positive PCR amplifications of several regions specific for the *Mycobacterium tuberculosis* complex and the presence of mycolic acid [3]. For determining the presence of ancient mycolic acid we use a sensitive analytical technique: Fourier transform infrared spectroscopy (FT-IR).

2. EXPERIMENT

The skeleton analyzed presents lumbar vertebral lesions, multiple vertebral bodies fusions and for these reasons we consider that the damage is most likely to be produced by tuberculosis, resulting in Pott's disease as a preferred diagnosis. After the anthropological and anthropometrical analysis, we proceeded with the DNA extraction using a variation of the phenol-chloroform protocol. In all steps of the experiment we maintained a sterile working environment [4].

The samples for the FT-IR analysis are taken from various parts of the skeleton. Bone powder samples weighting 0.8 mg were ground in an agate mortar with 150 mg KBr and compressed in a sample pellet. Infrared spectra were obtained between 400 and 4000 cm^{-1}. For each sample the KBr interferences were cancelled and baseline was corrected. After obtaining the infrared absorption spectrum for each sample we examined the results focusing on the specific absorption patterns of mycolic acid [5]. We selected the bones for DNA extraction based on the FT-IR results. Despite the issues raised by ancient DNA extraction from bones, we managed to get sufficient DNA for further analysis.

In our study we tried to amplify 4 regions from *Mycobacterium tuberculosis* genome. Primers were designed in order to produce ~100 bp amplicons. Because we

were working with ancient DNA, we were forced to get as much information as we can from short sequences. Therefore, our primers are designed to amplify: 1) *IS1081* element, a multi-copy locus characteristic for *Mycobacterium tuberculosis* complex, 2) *oxyR* locus which contains a SNP that distinguishes between *M. tuberculosis* and *M. bovis*, 3) *pncA* locus – for the same reason as the previous and the 4) D1 deletion - which has been used to differentiate evolutionary "old" and "modern" strains [2,6].

3. RESULTS AND DISCUSSION

Using FT-IR analysis we obtained spectra that contain some patterns specific for long-chain fatty acids such as mycolic acid: absorption peaks at 2960 and 2930 cm^{-1} corresponding with asymmetric C-H stretch of the methyl and methylene groups, and long chain deformation at 720 cm^{-1}. Those are not indisputable evidence of *Mycobacterium* presence, but can offer us guidance on what bones might have been infected with this pathogen. We use these data for selecting the bones for DNA extraction, and after PCR amplification we obtain conclusive result indicating the presence of the pathogen. Nevertheless, our findings must be confirmed by sequencing.

4. CONCLUSION

The most recent common ancestor of *M. tuberculosis* complex evolved approximately 40.000 years ago. The speciation of the members belonging to M. *tuberculosis* complex is supposed to have happened in various time moments according to each species. Using a case of tuberculosis from that period will help us observe the evolutionary state of this infectious agent.

If our results will be positive after verification by sequencing, this study could provide additional information regarding the evolutionary scenario for *Mycobaterium tuberculosis*.

5. ACKNOWLEDGEMENTS

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TESTING FOR TYPE 2 DIABETES IN AN ANCIENT HUMAN SKELETON USING MOLECULAR METHODS

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1. INTRODUCTION

Diabetes mellitus is a metabolic disease. The affected patients have high blood sugar levels because either the pancreas does not produce enough insulin or the organism cannot use the insulin due to insulin resistance.

Left untreated, diabetes can cause many complications. Most common are: diabetic retinopathy, diabetic nephropathy, diabetic neuropathy (contributes to diabetic related foot gangrene problems).

Because soft tissues are degraded post mortem, when using ancient human remains only the skeleton and teeth are available for anthropological and molecular studies. This is the reason why the diagnostic of diabetes is made based on certain osteopathological changes associated with diabetes. Parallel to the anthropological analysis, our objective was to confirm this diagnostic using molecular methods.

One skeleton from the Suplacu de Barcău (Romania) archaeological site, dated to the Neolithic period, displays pathological changes that indicate diabetes.

2. EXPERIMENT

The subject of this study are the skeletal remains of an adult male from the Suplacu de Barcău archaeological site dated to the Neolithic period (5000 years B.C.). More exact dating will be provided by radiocarbon dating.

Initial identification of diabetes mellitus was made through differential diagnosis, based on the presence of caries, ante mortem tooth loss, DISH (Diffuse Idiopathic Skeletal Hyperostosis), osteoarthritis, major lytic lesions on both feet affecting the calcanei.

This diagnostic is to be confirmed through molecular data. This is a very challenging task, because diabetes is a complex disease, which is caused by genetic factors as well as lifestyle and environmental factors.

Given the fact that the individual was a 40-45 years old adult at the time of death, we tested a set of gene polymorphisms associated with maturity onset diabetes mellitus. The selection of candidate genes was based on previous studies made on modern populations from various ethnic groups.

Glucokinase (GCK) is the key glucose phosphorylation enzyme. SNP *rs1799884* has been associated with MODY2 (Maturity Onset Diabetes of the Young 2). This mutation is frequent among MODY patients (aprox. 65% of all patients).

Another genetic factor predisposing to diabetes might be a polymorphism in the calpain 10 gene (CAPN 10). SNP *rs3792267* was previously associated with insulin resistance.

We also considered a mutation in mitochondrial encoded tRNA leucine 1 (MTTL1) associated with Maternally Inherited Diabetes and Deafness (MIDD).

Detection of the above mentioned mutation was made using PCR-based assays.

3. RESULTS AND DISCUSSION

The musculoskeletal symptoms considered together most likely indicate diabetes mellitus.

The molecular data was interpreted by comparison to healthy individuals from the same period and confirmed cases of diabetes from nowadays Romanian population.

4. CONCLUSION

The Neolithic period was characterized by the transformation of human societies from being hunter-gatherer based to agriculture based. We believe the presence of diabetes in this population is related to the lifestyle and diet changes.

5. ACKNOWLEDGMENT

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MITOCHONDRIAL HAPLOGROUP DIVERSITY IN A 10th CENTURY AD MEDIEVAL POPULATION FROM MIREASA, CONSTATA, ROMANIA

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1. INTRODUCTION

Research on 10th century populations from the South-eastern part of Romania is important due to the frequent migrations that occurred in the region during that period. Studying a large necropolis (Mireasa, Constanta) containing 150 skeletons can offer detailed information about their origin, lifestyle, patterns of genetic diversity or show signatures of population migrations and their influence on the native gene pool. An anthropological examination of each skeleton was required in order to register physical evidence of their activities or diseases. Additional information to the anthropological research was obtained working with ancient DNA, using various genetic markers, mainly belonging to the mitochondrial genome.

2. EXPERIMENT

Obtaining workable quantities of DNA from ancient human remains can be a challenge for any researcher. Postmortem degradation of DNA results in high fragmented and damaged copies. Also high calcium concentration and the presence of organic matter in bone or tooth may interfere with the reactions in which the extracted DNA will be used after extraction. In order to obtain high concentration of pure DNA a modified phenol-chloroform extraction protocol was used [1]. Another challenge was to avoid contamination with modern DNA, consequently standard anti-contamination protocols were followed [2]. Extracted ancient DNA fragments were amplified, cloned and sequenced to get mitochondrial HVR I (HyperVariable Region I). Identifying SNPs by comparison to the revised Cambridge Reference Sequence (rCRS) helped situating each individual to a mitochondrial haplogroup.

3. RESULTS AND DISCUSSION

Even if DNA was damaged and highly fragmented, workable quantities were extracted without any contamination. The DNA was extracted from different samples belonging to both male and female individuals of different ages in order to cover a larger span of the population. The classification of the resulting samples into their haplogroups allowed the researchers to clarify the origins of the population and also to assess the migratory impact on the native population.

4. CONCLUSION

The team of archaeologists that conducted the research at the necropolis from Mireasa had difficulties in precisely dating the majority of tombs especially due to lack of inventory found in the graves and therefore had to refer to other criteria such as the general orientation of the graves and several other analogies to the existing literature. The present research would in fact bring additional, more conclusive, information based on the DNA analysis of the samples that will help not only date the whole site under study but also increase the knowledge level about the members of the mediaeval community under study which is most likely dating from a historical age that is still a mystery for historians: the dawn of the Eastern European mediaeval ages.

5. ACKNOWLEDGMENT

This study was supported by funding from the project Genetic Evolution: New Evidences for the Study of Interconnected Structures (GENESIS). A Biomolecular Journey around the Carpathians from Ancient to Medieval Times. (CNCSIS-UEFISCDI _PNII_PCCA_1153/2011)

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MITOCHONDRIAL HAPLOGROUP DIVERSITY IN A 10th CENTURY AD MEDIEVAL POPULATION FROM CAPIDAVA, CONSTATA, ROMANIA

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1. INTRODUCTION

Recent advances in ancient DNA techniques have provided new insights on essential archaeological and anthropological questions solving several controversies regarding past human lifestyles from different time scales. Among the multitude of information rendered by ancient DNA analysis from human remains, data concerning human evolution, prehistoric human migrations, kinship and social organization in past societies is unravelled. In this light, genome investigation from human remains recovered from an archaeological site located in South-eastern Europe (Romania) can give valuable, additional information in order to complete the picture of patterns in the medieval history of South-eastern Europe. The most straightforward method to perform population genetic analysis is to use as molecular marker the hyper-variable regions of the mitochondrial genome so as to estimate mitochondrial haplogroup diversity [1]. The current study is part of an extended paleogenetic research centred on mtDNA polymorphisms in ancient populations from several key periods spanning from ancient to medieval times.

2. EXPERIMENT

The biological samples are represented by bones and teeth corresponding to 9 human skeletons excavated from a medieval burial site. The necropolis is located in Capidava, an archaeological 10th century site in the South-eastern part of Romania, near the Danube which was a key stop in the route of migratory populations [2].

The entire process of obtaining DNA sequences was carried out following standard procedures in order to overcome two main problems contamination with exogenous DNA and molecular degradation [3,4]. DNA was extracted using a phenol/chloroform protocol [5], with changes suggested in several other publications [6,7]. After the extraction protocol was carefully followed, the DNA was concentrated and purified by passing the elution through Centricon-30 columns, Amicon.

The amplification of the product was done in a separate room designated for polymerase chain reaction procedure, restricted to solely ancient DNA analyses Negative PCR controls are run with each PCR series and two blank controls for each extraction series. For each specimen, overlapping segments of the mtDNA control region's first hyper-variable region (HVR_I) were amplified by PCR [8]. We varied the DNA polymerase type (MangoTaq, MyTaq, PlatinumTaq) and the concentrations of some

reagents such as Mg^{2+} in order to find the best combination which leads to increased efficiency and reduced amplification errors. After reconstructing HVR_I the amplicons are cloned (CloneJet PCR Cloning, Thermo Scientific) and for each successful sample a minimum of five clones are sequenced (Macrogen, Netherlands).

3. RESULTS AND DISCUSSION

Taking into consideration the fact that the current study is still in development, we have obtained some essential results. In the first place, we observed that the DNA contained in teeth samples from ancient skeletons was better preserved in comparison to the one contained in bones of the same specimen. This suggests that bones are more subjected to diagenetic processes than teeth. In the second place, we developed efficient strategies to overcome main problems such as contamination with exogenous DNA or presence of PCR inhibitors by following standard experimental procedures. At the same time, we tested and adjusted all steps of the analysis. In this manner the amplification results were optimized and the necessity of an additional step in the extraction protocol of degraded DNA was confirmed (use of Centricon filters). Moreover, by analyzing the mtDNA sequences for every individual recovered from Capidava supplementary information regarding population dynamics and phylogeny will be given. Therefore, genetic data might fill in the gaps of historical and archaeological information regarding the investigated population which probably belongs to the group of the pechenegs which were settled in South-eastern Romania, during the 10^{th} century, including in Capidava.

4. CONCLUSION

To conclude, in the current study we genetically investigated an ancient population that consists of 9 skeletal remains, dating from the early medieval period. We managed to obtain a reliable and adequate amount of ancient DNA so as to establish the haplogroup diversity of this population excavated from Capidava.

5. ACKNOWLEDGMENT

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A CORRELATION BETWEEN PHYSICAL ANALYTICAL METHODS AND THE RATE OF DNA EXTRACTION FROM ANCIENT HUMAN REMAINS

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1. INTRODUCTION

DNA is gradually degraded after inhumation, thus making the extraction of it a sedulous labour with a low yield, while consuming expensive reagents. Our work is focused on finding a correlation between bone properties that are easily accessed through physical techniques, such as Fourier Transform Infrared Spectroscopy (FT-IR), SEM, XRD and the quantity and quality of the extracted DNA. This study compares data about different types of bones (long, short, flat), of compact and trabecular tissue, from individuals estimated to range from newborns to elders, and dated from the Bronze Age to the Medieval times. The result should be an index to help choose the most appropriate samples for further processing, *i.e.* DNA extraction.

2. EXPERIMENT

The remains were anthropologically and anthropometrically analysed for estimation of gender and age of individuals [1, 2]. Various bones were selected for physical analyses: diaphyses and epiphyses, skull fragments, vertebrae bodies, carpals and tarsals, ribs.

A very small amount of bone is necessary for the physical analyses, *e.g.* 0.8 mg of bone shaving for the FT-IR spectroscopy, which is ground into a fine powder and mixed with potassium bromide (KBr), then pressed into a pellet. IR spectra are obtained for wavelengths between 4000 and 400 cm^{-1}. The organic content of the sample, mostly collagen, should be proportional to the DNA in the tissue and it is measured from the Amide I peak at 1585-1720 cm^{-1}. The inorganic fraction of the bones, the hydroxy-apatite, is assessed from the phosphate and carbonate peaks at 900-1200 cm^{-1} and 850-890 cm^{-1} respectively [3,4].

The DNA extraction requires a more significant amount of bone shavings, 200 mg. Using a variation of the phenol - chloroform protocol [5], genomic DNA is extracted and the quantity and quality are assessed by electrophoresis in agarose gel and spectrophotometry. The entire process takes place in a sterile environment, and several controls are done with every extraction [6].

3. RESULTS AND DISSCUSION

FT-IR spectra for many samples were analysed, comparing intra- and inter-individual types of bones, adult, elder and infant tissues, and remains from different

historical ages and with different levels of diagenesis. The peak area of Amide I shows the amount of peptide links in the composition of proteins, here collagen.

An example of genomic DNA extraction results is shown in Fig. 1. The smear observed in the samples lanes are consistent with the fact that DNA is highly degraded in ancient human remains, but the highest bands indicate that there still is undegraded DNA. The controls denote the lack of contamination, and each sample is tested by another colleague for bacterial DNA in order to verify if the genetic material extracted is human or there are bacterial species in the bones.

4. CONCLUSION

Physical characteristics of ancient human bones and other information that is easily obtained can be correlated to the extent of degradation and of course, conservation of the DNA, thus allowing us to choose the most appropriate bone from the remains for the extraction protocol, saving us the time and reagents spent on nibbling various unlucky parts of the skeleton.

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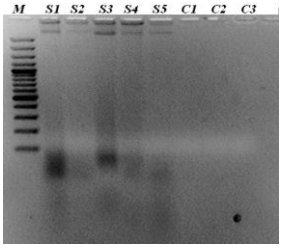

Fig. 1. *Gel electroforesis of genomic DNA extracted using the modified phenol-chloroform protocol. (M=100 bp marker, S=sample, C=control).*

ECOLOGY AND ENVIRONMENTAL SCIENCE

POTENTIAL USAGE OF ESSENTIAL OILS AS FUNGICIDES IN CULTURAL HERITAGE CONSERVATION

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1. INTRODUCTION

Fungi are capable of colonizing, degrading and altering a variety of materials, which have been used through the centuries for creating cultural heritage monuments and artifacts causing biodeterioration [1]. Essential oils (EO), as natural products, can be used as environmentally friendly alternative to commonly used biocides [2]. Antimicrobial activity of many EOs has been reviewed by Kalemba and Kunicka [3], and many EOs have been successfully applied in different fields of microbiological control. But reports regarding the use of EOs as fungicide in cultural heritage conservation are very scarce. The aim of this study was *in vitro* to evaluate antifungal potential of *Origanum vulgare* L. EO against fungi isolated from cultural heritage objects.

2. EXPERIMENT

Fungi tested in antifungal assay were isolated from different cultural heritage objects, stone monuments and wooden sculptures (Tab. 1).

Qualitative and quantitative analyses of the *O. vulgare* EOwere performed using GC and GC-MS. Microdilution and microatmosphere methods were used for testing the antifungal activity of EO, while biocide Benzalkonium chloride (BAC), used as positive control, was tested using micro- and macrodilution methods. BAC belongs to quaternary ammonium compounds which are approved for conservation of cultural heritage monuments by the European Biocide Directive as relatively environmentally friendly biocides [4].

3. RESULTS AND DISCISSION

High fungistatic and fungicidal activity of *O. vulgare* EO was demonstrated with low MIC and MFC values, ranging from 0.1 to 2 μL mL-1, obtained in both methods used (Fig. 1). The isolate most susceptible to EO treatments was *E. nigrum*, while the *T. viride* was the most resistant. BAC confirmed its strong antifungal activity against the tested fungal isolates (Fig. 1). Tested *Aspergillus* species were the most susceptible to BAC treatment.

4. CONCLUSIONS

Obtained results confirmed the well-known efficacy of low concentrations of biocide BAC. Strong antifungal activity of *O. vulgare* EO against tested fungi suggested the potential usage of EOs as novel fungicides in cultural heritage conservation. However, further studies are required to develop appropriate methods to apply EOs in conservation of cultural heritage objects.

According to Saxena and Mathela [5] EOs could be good alternatives for existing biocides due to their low mammalian toxicity, susceptibility to biodegradation and strong antimicrobial activity.

5. ACKNOWLEDGEMENT

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Tab. 1. Tested fungal isolates.

Fungi	Substratum
Aspergillus niger Tiegh	wooden sculptures
Aspergillus ochraceus G. Wilh	
Penicillium Link sp.	
Trichoderma viride Pers.	
Bipolaris spicifera (Bainier) Subram	stone monuments
Epicoccum nigrum Link	

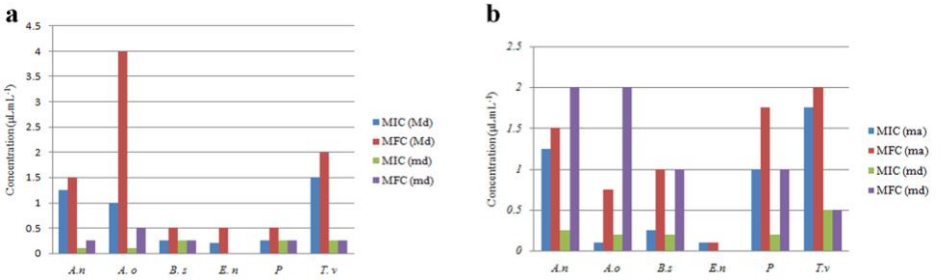

Fig. 1. *Antifungal activity of BAC (**a**) and O. vulgare (**b**): A, niger (A.n), A. ochraceus (A.o), B. spicifera (B.s), E. nigrum (E.n), Penicillium sp. (P) and T. viride (T.v); Minimal inhibitory concentration (MIC); Minimal fungicidal concentration (MFC); microatmosphere method (ma); microdilution method (md); Macrodilution method (Md).*

JOINT DANUBE SURVEY 3: MICROBIOLOGICAL QUALITY AND GENOTOXICITY ANALYSIS

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1. INTRODUCTION

The Joint Danube Survey 3 (JDS3) is the world's biggest river research expedition of its kind in 2013. The JDS is carried out every six years – JDS1 was in 2001 and JDS2 in 2007. For six weeks between 13 August and 26 September, the JDS3 ships travelled 2,375 km downstream the Danube River, through 10 countries, to the Danube Delta. An international Core Team of 20 scientists consisted from experts for microbiology, macrophytes, phytoplankton, phytobentos, macrozoobentos, fish and chemistry [1].

To be safe for consumption, water must be free of pathogenic bacteria among which enteric pathogens are the ones most frequently encountered. While total coliforms only arise suspicion on faecal pollution in aquatic environments, the faecal coliforms indicate presence of faecal pollution with high probability; they are mainly represented by *Escherichia coli* and together with faecal streptococci are used widely in examination of the water quality.

The simple detection of pollutants in environment provides only limited data on the substances present in the environment and gives no information on the relationship between contaminant exposure and biological effects in aquatic organisms; therefore a proper evaluation of the impact of pollutants by biomarkers becomes essential. Mussels and fish are commonly employed in biomonitoring; nevertheless our previous studies confirmed their applicability for studying DNA damage as biomarker [2,3,4].

The comet assay is a relatively simple procedure which evaluates DNA damage by the tail fluorescence intensity of single gel-embedded cells following alkaline electrophoresis. The ability to make a measurement of damage at the genetic level

provides information relevant to determining the health of the environment, particularly in the context of investigative monitoring.

2. EXPERIMENT

Indicators of faecal pollution were isolated using the Colilert® and the Quanti-Tray/2000. The Colilert® simultaneously detects total coliforms and *E. coli* density using the nutrient indicators *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and 4-methylumbelliferyl-β-glucuronide (MUG), which is metabolized by total coliforms and *E. coli*, respectively. Detection of enterococci was performed with Biorad kits according to ISO 7899-1 (1998; multiwell plates, MPN technique).

Comet assay was performed on haemocytes of mussels *Unio pictorum*, *U. tumidus* and *Sinanodanta woodiana* and erytrocytes of fish *Alburnus alburnus* and *Neogobius melanostomus.* Total of 217 specimens of mussels and 98 of fish were analysed.

3. RESULTS AND DISCUSSION

The results of microbiological analyses are still being processed. Currently, results indicated similarities with the results of JDS2 in presence of hotspots of faecal pollution and influences of pollution in tributaries on the level of pollution of the Danube River. The results of genotoxicity analysis indicated presence of genotoxic pollution at some parts of the river. The highest levels of DNA damage were detected in animals collected in upper and middle course, while the lowest level of DNA damage was detected in animals from lower course of the Danube River.

4. CONCLUSION

To conclude, our results indicated presence of faecal and genotoxic pollution in the Danube River. However, it should be kept in mind that our results are obtained by single measurement and represent snapshot of the Danube River current condition.

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MARINE MAMMAL AND HUMAN INTERACTIONS IN THE MEDITERRANEAN SEA

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1. INTRODUCTION

The term "marine mammals" describes that group of mammals that depend on the marine environment as a source of food. This group is comprised mainly of cetaceans and seals, 11 species of which are regularly found in the Mediterranean, along with 4 occasional species and 8 vagrant ones [1].

Monitoring these animals is a costly, laborious task, involving either direct observation or the use of tracking devices. However, with the advancement of technology and the popularity of social networks, there is plenty of information available online. One aspect of this project was to assess the social network YouTube as a source of such information. The other aspect of human-marine mammal interaction is not positive. These animals have long been considered pests by fishermen, inasmuch as they compete for similar species of fish [2]. This negativity is evidenced by the fact that both seals and dolphins in the Mediterranean were deliberately killed in large numbers until recently. Further, fisheries are responsible for the depletion of fish stock as a food source for marine mammals, as well as their accidental killing [3]. In the interest of solving this problem, it is necessary to determine the magnitude of damage done to both sides.

2. EXPERIMENT

For the first part of this study, over 200 videos were found on YouTube, using keywords such as "marine mammals", "dolphins", "whales", "seals" and "Mediterranean" or "Aegean" in various languages. Only those which recorded sightings of marine mammals in the wild were used. Specifically, 50 videos were further analysed, and data such as the location and date of the sighting, the species and the number of animals sighted etc., were recorded. This information was depicted on a map of the Mediterranean, as well as one of the Aegean Sea.

For the second part of the study, a total of 30 interviews was conducted with coastal fishermen in four fishing-ports in the North-western Aegean. The questionnaires covered such topics as, the gear types used, the most frequented fishing areas, the species of marine mammals sighted, the cost of the damage caused, the seasons with most sightings and highest damages and the finding of dead or wounded cetaceans.

3. RESULTS AND DISCUSSION

The analysis of the videos show that the most frequently sighted species in the Mediterranean are the bottlenose (*Tursiops truncatus*) and short-beaked common (*Delphinus delphis*) dolphin, while those with fewest sightings are the humpabck whale (*Megaptera novaenglia*) and Risso's dolphin (*Grampus griseus*). This is most likely due

to the fact that the smaller dolphins are coastal, and therefore easily observed from small boats, whereas most other species prefer offshore, deeper waters [1]. In the Aegean, the most frequently sighted species are the bottlenose and striped (*Stenella coeruleoalba*) dolphin. There are no records of Mysticeti sightings, due to the aforementioned reasons. Also, the number of striped dolphin sightings is twice as high in the Southern Aegean as that in the North. A slight dip in the number of recorded sightings is noticeable during the years 2009-2010, and may be attributed to a drop in bottlenose and common dolphin density in that period. It must be noted that several videos are of poor quality, making it impossible to identify the species involved, and that the location and/or date of the sighting is often not mentioned.

As to the questionnaire results, despite the varying characteristics and habits of the fishermen, the depredation of nets is constant, and the attitude towards marine mammals is mainly negative. Conservative estimates of the damage cost range from 1000 to 13000 euros/year. The southernmost study area has the highest overall damages, but there is no correlation of damage cost with other characteristics. Species involved are most often bottlenose dolphins, as well as common and striped dolphins and monk seals (*Monachus monachus*). The number of seals is higher in the southern study areas, which are close to the largest resident seal population in the Mediterranean, while striped dolphins are absent from the last one, which is an enclosed gulf. The higher damages are possibly attributable to the presence of more seals in the third area.

4. CONCLUSION

The use of YouTube as a source of data on the distribution and density of marine mammals is possible. The drawbacks include occasionally poor video quality and a lack of location and date. However, despite the small number of videos included in this study, the overall distribution of cetaceans seems to corroborate existing bibliography [1,4], as well as the reports of the interviewed fishermen. A follow-up project would provide further insight and more complete information.

The problem of marine mammal and fisheries interactions is constant, and not affected by boat or engine size, or gear type. However, it has not been fully studied, and requires, among other things, a more accurate method of estimating the cost of damages, detailed reports of interactions and better species identification.

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NEW TAXON OF THE GENUS *NAVICULA* (BACILLARIOPHYCEAE) FOR THE DIATOM FLORA OF SERBIA

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1. INTRODUCTION

Diatoms are large and diverse group of single-celled algae [1]. They are distributed throughout the world in nearly all types of aquatic systems and are one of the most important food resources in marine and freshwater ecosystems [2,3].

The main objective of this paper is to report benthic, epilithic diatom species from the Raška River. First time Husted described *Navicula jakovljevicii* in 1945 [4]. This taxon has been found outside the Balkan area for the first time, in the Lake Zug, Switzerland [5]. Until now, there is no published data about diatom flora in Raška River. In the present study, we sampled benthic diatoms to evaluate the floristic richness of the river.

2. EXPERIMENT

The material was collected in April, June, August and November 2011 and March and May 2012 from 5 localities along the Raška River. Epilithic samples were scraped from the surface of stone by brush. Samples were fixed immediately with formaldehyde to a final concentration of 4%. Samples were treated with concentrated sulphuric acid and potassium permanganate [6]. Light microscope observations and micrographs were made using the Zeiss AxioImager M.1 microscope. Terminology of valve morphology is based according Hofman & Lange-Bertalot [7]. The abundance was estimated by counting 400 valves of each taxa present on slide.

3. RESULTS AND DISCUSSION

The paper present the description and distribution of the new species for the diatom flora of Serbia and its ecology.

Navicula jakovljevicii Hustedt 1945.

Description: Valves lanceolate to elliptic-lanceolate with obtusely to broadly round ends, 36.06-70.08 μm long, 8.53–11.46 μm wide. Raphe fissures weakly lateral, more or less distinctly curved. Axial area very narrow, linear, central area small, weakly asymmetrically rounded. Striae moderately lateral, parallel to weakly convergent at the ends, 15–16/10 μm. **Ecology**: According to Lange-Bertalot [8], the species prefer waters which are calcium carbonate buffered and oligo- to eutrophic. Total hardness at the sampling sites was 31.58-31.96 mg $CaCO3/dm^3$. Torrisi & Dell'Uomo [9] reported that *Navicula jakovljevicii* is alkaliphilic species. Our data showed that water is alkal (7.7-7.85). Water temperature range from 11.05 to 11.53°C, conductivity was moderately

with low concentrations of nutrients. **Distribution (Serbia)**: *N. jakovljevicii* was found in June, August and November 2011 and March and May 2012 along the flow of the investigation, in relatively low abundance (0.5 %). **Distribution (Europe)**: South-east and central Europe, (ex) Yugoslavia, the Lake Zug (Switzerland), the River Ager, a tributary of the Traun River (Austria) [8], France [9], central Apennine rivers [10], and Macedonia [11].

4. CONCLUSION

Navicula jakovljevicii is a new species to the Serbian diatom flora. Evaluation of the floristic richness of diatoms in the rivers is a necessary, further step. This new information increases our knowledge of the river system and chemical parameters, which is important for further predictions of diatoms as bio-indicators, and monitoring [12].

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WORKSHOPS

MicroRNA DATA ANALYSIS: A SPECIAL FOCUS ON CONSOLE APPLICATIONS

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WORKSHOP SUMMARY

MicroRNAs (miRNAs) are small (~22 nt long) non-coding RNA molecules which have been identified as important regulators of gene expression. The initial step in their biosynthesis is the transcription of miRNA genes by RNA polymerase III [1] or the transcription of intergenic regions by RNA polymerase II [2] which lead to the formation of the pri-miRNA structure. The latter steps include processing by the enzyme Drosha, the formation of the pre-miRNA stem-loop structure (hairpin), Exportin 5 mediates the transfer to the cytoplasm where the enzyme Dicer cleaves the terminal loop, and finally separates the two RNA strands, which leads to the formation of the mature miRNA. The mature miRNA functions by RNA Induced Silencing Complex (RISC) [3] mediated binding to its target mRNA transcript. This step is the most important step in the regulatory pathway of miRNAs and it is believed that it usually takes place at the 3'-UTR of the target transcript and causes gene silencing or degradation [4]. However, increasing evidence is being presented suggesting that miRNAs may target the 5'-UTR and coding regions, too [5-7] where they could be responsible for an increase in the expression levels [7-11].

Since the identification and targeting of miRNAs are both time consuming and costly, computational efforts have been initiated to ease the process. Many such bioinformatics applications come from master and PhD work done by students aiming to graduate and move on. This causes many problems when we set out to use these programs as they usually lack GUIs and proper documentation.

In this workshop we aim to present a number of current computational approaches in miRNA data analysis ranging from miRNA gene prediction to target prediction. Both programs which have GUIs and those which are console based will be covered and we will have a special focus on the efficient use of the console.

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FEEDING THE INCREDIBLE BRAIN

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WORKSHOP SUMMARY

While processing the information that comes from of the body sensation systems, brain produces regulatory responses to all of the body's organs and tissues. Understanding the brain is extremely hard, if even possible, task. Still we will try to take a look "inside" our sensational neural networks to see some interesting properties and features of its work.

A number of substances interact with neural system. Some brings good consequences, some – bad. So here we shall discuss about food for our brain.

TARGETING CANCER METABOLISM: OLD STORY, NEW ANGLE

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WORKSHOP SUMMARY

For production of energy normal cells rely on mitochondrial oxidative phosphorilation, whereas cancer cells tend to shift to non-oxidative production of ATP; this phenomenon has been termed the Warburg effect [1]. Herein, we discuss the view on Warburg effect as a primary cause for cancer or as a consequence of cancer pathogenesis. Structural and functional changes within the mitochondrial cell compartment are closely linked to Warburg effect and to cancer initiation. New therapeutic methods have been developed thanks to knowledge we possess on cancer cell metabolic shift, and some new selective drugs are in development [2]. This workshop is focused on past, present and future of Warburg effect with in-depth scope on biochemical, molecular, cellular and therapeutic aspects.

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THE FIRST HUNDRED YEARS OF MICHAELIS-MENTEN ENZYME KINETICS

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WORKSHOP SUMMARY

It has been a full century since the foundation of the famous enzyme kinetics model, set by Leonor Michaelis and Maud Leonora Menten [1]. The equation commonly called the Michaelis–Menten equation (Equ. 1) was first set in 1913, using invertase ("*invertin*") as the experimental model [2]. It was a time when the scientists did not even know for sure what biological molecules actually carry out the catalytic function of "ferments".

Since then, much has changed and many discoveries made us think differently in terms of molecular biology as a fast growing science. But, impressively, this model survived to celebrate a full century of existence, being only complemented, not ever changed in its essence.

Some of the latest contributions in the field gave a new dimension and opened up some novel perspectives [3].

This workshop shall include a brief talk, fishbowl discussion on the spot and, if feasible, computer simulations.

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$$V = \frac{Vm \times [S]}{Km + [S]}$$

Equation 1. *Michaelis-Menten equation (labelled as it is nowadays); V - the velocity of the enzymatic reaction, Vm - maximum (plateau) velocity, [S] - substrate molar concentration, Km - Michaelis constant.*

AUTHOR INDEX

SOYBEAN PHYTOESTROGENS AND MENOPAUSE-FRIENDS OR FOE?

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Phytoestrogens are plant-derived compounds with potential estrogenic biological activity. They have a phenolic A ring, which enables binding for estrogen receptor (ER) [1]. Genistein (G) and daidzein (D) are a major phytoestrogens found in soy and soy based products [2]. It is structurally similar to estradiol and binds to both types of estrogen receptors, with higher affinity for ERβ. In addition, by exhibiting antioxidative and tyrosine kinase/steroidogenic enzyme inhibiting effects, phytoestrogens appear as alternative therapeutics for various ageing-related diseases [3]. Cessation of a women's reproductive function, *i.e.* menopause, is often accompanied with disorders that are consequence of reduced ovarian function and low estrogen concentration. Therefore, one of the possible therapeutic applications of genistein and daidzein is prevention and alleviation of menopausal symptoms, as an alternative to hormone replacement therapy.

The aim of this study is to examine the effects of phytoestrogens on the reproductive system of middle-aged female rats. Of particular interest is to define the potential of phytoestrogens for improvement of reproductive system function in an animal model of menopause, and to compare these effects with the effects of therapeutic doses of estradiol dipropionate (E), commonly used in prevention and treatment of menopausal symptoms.

Middle-aged, 12 months old, female rats subcutaneously received 35 mg/kg of G or 35 mg/kg of D dissolved in a mixture of olive oil and ethanol (9:1), daily, for 4 weeks. Also, female rats subcutaneously received 0.625 mg/kg of E, dissolved in olive oil. Each of the treated groups had a corresponding control group. Thus, females of the control phytoestrogen groups (CP) received sterile olive oil and ethanol, while those of the E control group (CE) were given sterile olive oil. Intact control group (C) was also established. Each group consisted of five animals. All animals were sacrificed 24 h after the last injection.

Chronic estradiol treatment of middle-aged female rats caused an inhibition of gonadotropic cells, which was reflected not only in the reduction of fluorescent signal intensity, but also in reduction of the cell size. At the same time, estradiol stimulated lactotrops, by increasing their density and the relative intensity of the immunolabeled prolactin in cells. In contrast to estradiol treatment, genistein and daidzein did not cause changes in the relative intensity of the fluorescent signal within the gonadotropic or lactotropic cells. However, changes in cell morphology were observed after treatment with both phytoestrogens. Gonadotropic cells were larger in size, while lactotropic cells were smaller comparing to the controls. All types of examined pituitary cells were changed in shape with unevenly stained cytoplasm, that is, immunolabeled parts of cytoplasm were separated by unstained regions, which gave cells the appearance of vacuolization.

Chronic treatment with therapeutic doses of estradiol, in the ovaries of acyclic females, generally had a negative effect. In the group of small follicles, estradiol caused a significant increase of atretic, primordial, primary and preantral follicles. Similar effect was observed in the group of antral follicles. Estradiol treatment caused an increase of volume and number of corpora lutea, which resulted in the ovary volume increase. In contrast to estradiol, chronic application of genistein and daidzein caused a significant increase in the number of follicles in initial stage of folliculogenesis (primordial and primary follicles), without affecting total number of atretic primordial follicles, but significantly reducing the number of primary atretic follicles. Treatments with phytoestrogens did not affect total number of preantral and antral follicles, while they caused a decrease of atretic antral follicle number. Genistein treatment did not change the number of individual classes of corpora lutea, or their total number. Unlike genistein, daidzein increased the number of mature corpora lutea, which resulted in an increase of their total number, and ovary volume. However, due to the preservation of healthy follicles, both treatments caused an increase of follicular parenchyma volume.

Estradiol caused decrease of ERα mRNA and increase of PR mRNA expression, while ERβ mRNA expression was not changed. Genistein caused an increase of uterine weight and endometrial volume density, decrease of ERα mRNA expression, and increase of PR and ERβ mRNA expression. In contrast to genistein treatment, daidzein did not change uterine weight, or morphometric and stereological characteristics of uterus. Expression of ERα and PR mRNA was not affected, while expression of ERβ mRNA was increased.

In vagina, application of estradiol caused hypertrophy of the epithelium, followed by desquamation of epithelial cells. After treatment with phytoestrogens, changes in general histological appearances of the vaginal epithelium were not observed. However, detailed light microscopic analysis revealed less numerous pyknotic nuclei, and more numerous mitotic cells.

Comparing to estradiol, genistein and daidzein, exhibited numerous beneficial effects on the reproductive system of middle-aged females, especially in the ovary. Bearing in mind that menopause, among other things, is caused by reduced ovarian function, the results of this study are significant and applicable, given that the preservation of ovarian function can significantly improve the quality of life of women. Therefore, it can be concluded that genistein and daidzein could be a successful alternative to estrogen replacement hormone therapy.

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THE PLASTIC DIET: WHAT IS HAPPENING TO SEA TURTLES?

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1. PLASTICS IN THE MARINE ENVIRONMENT

Marine debris is a growing problem in our consumer society. It is estimated that 80% of marine debris is some forms of plastic, adding up to approximately 100 million tons of plastic distributed throughout the oceans. This vast amount of plastics could cover an area of USA and India combined [1]. Yearly production of plastics has been steadily growing since the 1950s. Use of plastic materials has reached 100kg per capita in North America and Western Europe, and is predicted to reach 140kg per capita in 2015 [2]. Cups, bags, bottles, balloons, packaging and other objects reach the oceans by rivers, storms, or by direct littering, and are then carried by ocean currents to even the most remote islands and ocean areas. If left in the oceans long enough, larger plastic items are broken down into micro-particles [3]. In terms of mass, micro-plastics are in some areas significantly more abundant than plankton [4]. This accumulation of (floating) plastic debris occurs in areas of large cyclonal currents, such as the Pacific and North Atlantic gyres [4,5]. Because of nutrient abundance, these are usually also feeding areas for many marine species. As the abundance of plastic debris is increasing both in the gyres and in the remote areas, so is its effect on the marine ecosystem [3].

2. INTERACTIONS WITH MARINE WILDLIFE

Plastics in the marine environment can affect marine organisms in various ways: entanglement; ingestion; transfer of toxicants and invasive species; and accumulation and destruction of benthic communities. Interactions with plastics have been documented in over 267 marine species, ranging from filter-feeders and planktivours species to fish, seabirds, sea turtles, pinnipeds and cetaceans [6]. As a consequence of entanglement or ingestion of plastic debris, over 100 million marine animals are killed each year [1]. The number of more subtle consequences of ingestion (e.g. reduced appetite and growth, reproductive complications, damaged digestive system, accumulation of persistent toxicants transferred by micro-particles) is even larger [6]. This effect will become predominant as the plastic marine debris particles are in most cases smaller than 2.5mm, and are decreasing as has been noted comparing the 1990s to the 2000s [7].

3. WHY WOULD SEA TURTLES EAT PLASTICS?

Archie Carr, one of the biggest sea turtle experts, noted: “Sea turtles are peculiarly prone to eat plastic scraps and other buoyant debris” [8] They are opportunistic carnivores and will eat almost anything, including plastic debris [9,10]. Plastic debris has

been found in digestive system of sea turtles worldwide: e.g. south Atlantic [11, 12], North Atlantic [5, 13], Mediterranean [10] and Adriatic [14]. In a long-term study on leatherback turtles, 34% of the necropsy reports mention plastic debris being present in the digestive system, its occurrence increasing from 1968 onwards [15].

It is physiologically impossible for sea turtles to regurgitate swallowed items. Swallowed plastic can block or damage the digestive system [12]. Furthermore, ingested pieces of plastic can stay in the digestive systems for months, and gases that are the result of harmful debris decomposition can stay trapped in sea turtles, making them buoyant. This makes it impossible for them to hunt, while making them an easy target for predators [1]. Often as much as 1-2 g of plastic is enough to cause sea turtle's death [10]. Most of the ingestions do not have an immediate lethal effect and do not cause physical damage to the gut itself [10], but the sole ingestion of plastic debris reduces the stomach/digestive system volume, resulting in less ingested and/or digested food. This effectively dilutes ingested food, and since sea turtles do not eat more food to compensate for dilution [16], this could affect the fitness of individual turtles and whole populations. Taking into account the long life cycle of sea turtles (sexual maturity at 20 years of age, and lifespan of 60 years), and their habitat (ocean realms), experiments are hard to conduct. This is where mathematical models and computations become very useful, if not necessary. I use one of such models to explore what could be the consequences of plastic ingestion on growth, maturation and reproduction of loggerhead turtles.

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